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THE LIPID COMPOSITION OF MICROCOCCUS HALODENITRIFICANS AS INFLUENCED BY SALT CONCENTRATION[1]

M. KATES, S. N. SEHGAL,[2] AND N. E. GIBBONS

Abstract

Cells of *M. halodenitrificans* grown in 1.0 *M* NaCl contained 11 ± 2% total lipids, of which about half was phosphatide. Seven lipid components were detected by chromatography, two of which were tentatively identified as phosphatidyl ethanolamine and phosphatidyl glycerol; lecithin was completely absent. No qualitative changes were found in the phospholipid or fatty acid constituents of cells grown in media containing different concentrations of sodium chloride, but cells grown in low salt concentration (0.6 *M* and 0.55 *M*) contained 7 to 10 times more unsaponifiable matter than cells grown at the optimal salt concentration (1.0 *M*). However, the addition of calcium, magnesium, or potassium to low-salt media restored the amount of unsaponifiable matter in cells to normal.

Introduction

Micrococcus halodenitrificans grows as a normal coccus in media containing 1.0 *M* NaCl, but cells grown in 0.6 *M* or 0.55 *M* NaCl without additional ions are generally distorted, swollen, and very sticky (19). Addition of 0.01 *M* calcium or magnesium ion was found to restore the cells to their normal shape; higher concentrations of potassium ion (0.3 *M*) achieve a similar effect (19). Cells grown at low salt concentrations were also found to synthesize less cell wall material (e.g. diaminopimelic acid) than normal, and the observed morphological changes were considered to result, at least partly, from failure of the cells to synthesize new cell walls during division (19).

In a previous study, normal cells of *M. halodenitrificans* were found to contain about 6% of lipid, associated largely with the cell wall (18). No information is, however, available concerning the chemical nature of the lipids of normal cells or of cells grown at low salt concentrations. The lipids of *M. halodenitrificans* grown at various salt concentrations were therefore investigated to determine whether any correlation existed between the observed morphological changes and lipid composition.

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Materials and Methods

Micrococcus halodenitrificans was grown on a rotary shaker in media containing 0.5% each of Proteose Peptone (Difco) and Tryptone (Difco), and the concentrations of sodium chloride and other salts indicated in Table I. After 72 hours' incubation at room temperature, cells were harvested by centrifugation and washed twice in sodium chloride solutions of the same concentration as the growth medium.

Extraction of Lipids

A water suspension of a known weight of cells (on dry weight basis) was poured with stirring into an amount of boiling isopropanol (referred to as one volume) sufficient to give a final concentration of 80% of isopropanol. The mixture was kept overnight at 4° C, then warmed to 60° C in a water bath and centrifuged. The insoluble residue was extracted once with 0.5 volume of hot isopropanol, twice with 0.5 volume of 1:1 chloroform–isopropanol mixture, and finally with 0.2 volume of chloroform. All centrifuged extracts were combined and concentrated at 35° *in vacuo* almost to dryness. To the lipid residue was added 0.5 volume each of chloroform and water, and the mixture was swirled gently. The emulsion formed was broken by addition of small amounts of a saturated sodium chloride solution and of a chloroform–methanol (10:1, v/v) mixture. After it had been held overnight at 4° C, the clear chloroform layer was removed and the aqueous phase was washed several times with small amounts of chloroform–methanol (10:1). Further washing of the chloroform phase with water was avoided because of the intractable emulsions formed. The combined chloroform extracts were concentrated *in vacuo* to a gelatinous mass, absolute ethanol and benzene being added to remove traces of water. The residue was redissolved in chloroform and portions of this solution were taken for the various analyses. In some experiments, cells were extracted with chloroform–methanol according to the procedure of Bligh and Dyer (3); similar results were obtained by both extraction procedures.

Analytical Methods

Ash-free dry weights were determined as described previously (18). Phosphorus was determined by the method of King (12), nitrogen by the micro-Kjeldahl method, sodium and potassium by flame photometry, and choline by the reineckate procedure (10). Total fatty acids and unsaponifiable material were determined as follows: the sample (50 mg) was saponified with 1 ml of 1 *N* NaOH on a steam bath for 2 hours; the mixture was acidified with 1 *N* sulphuric acid and extracted several times with a mixture of low-boiling petroleum ether (b.p. 40–60°) and ethyl ether (1:1). The extracts were concentrated in a stream of nitrogen, the residue was titrated in 3 ml of 90% methanol with 0.02 *N* NaOH in 90% methanol to the *o*-cresol red end point, and the unsaponifiable material was extracted with low-boiling petroleum ether. Fatty acids were recovered from the acidified methanolic phase by extraction with petroleum ether.

Fatty acid mixtures were methylated with diazomethane in ethyl ether and analyzed by gas–liquid partition chromatography. Early analyses were

made with a Podbielniak "Chromacon" using a 6-ft column of Celite impregnated with butanediol–succinate polyester (5) as stationary phase (column temp., 220°) and helium as the carrier gas (flow rate, 80 ml/minute). Later analyses were made with a "Pye Argon Chromatograph" using a 4-ft column of 1% polyethylene on powdered glass or Apiezon M on Celite (1:9, w/w), and argon as the carrier gas. The flow rate and temperature conditions were 100 ml/minute and 200° with the polyethylene liquid phase, and 107 ml/minute and 182° with Apiezon M. For both instruments the areas under the peak on the tracings were proportional to the molar concentration of the fatty acids in the mixture. Identification of the fatty acid methyl esters was made by comparison of their relative retention times with those of standard esters (9).

The polymer of β-hydroxybutyric acid was determined on an aliquot of lipid solution containing approximately 50 mg lipid. The chloroform was evaporated under nitrogen and the dry lipid was hydrolyzed in 0.2 *N* NaOH overnight at room temperature. The residue was washed once with 0.2 *N* NaOH and once with dilute acetic acid. It was then extracted with a mixture of petroleum ether – ethyl ether (1:1). The β-hydroxybutyric acid polymer was extracted from the residue with chloroform. The compound extracted was characterized by the formation of crotonic acid on hydrolysis and by carbon and hydrogen analysis (found: 55.8% C, 7.18% H; calcd.: 55.5% C, 7.3% H).

Chromatography of Lipids

Total lipids were analyzed by chromatography on silicic acid impregnated Whatman 3MM paper, using diisobutyl ketone – acetic acid – water (40:25:5) as solvent, following the procedure of Marinetti *et al.* (14). The chromatograms were stained with Rhodamine 6G (14) to detect total lipids, with ninhydrin reagent to locate amino-lipids, and with the periodate–Schiff reagent to detect vicinal glycol containing lipids (11).

Deacylation of Lipids

Total lipids were deacylated by mild alkaline hydrolysis following the procedure of Dawson (6), as modified by Maruo and Benson (16). The water-soluble phosphate esters in the hydrolyzates were analyzed by one-dimensional chromatography on Whatman No. 1 paper, using saturated phenol–water (PW; pH adjusted to 5.4 with 2 *N* NaOH), and butanol – acetic acid – water (BAW, 5:3:1). The phosphate esters were detected with the ammonium molybdate – perchloric acid reagent (4), and were identified by comparison of their R_f values in the two solvents with those of authentic compounds (2, 11, 16).

Results

Changes in Lipid Composition

Cells grown in 1.0 *M* NaCl (H_1) had a total lipid content of 11 ± 2% and a total phosphatide content of 5.5 ± 0.1% (Table I). These values were not significantly different for cells grown at lower salt concentrations (H_2, H_3), or at 0.6 *M* NaCl plus Ca^{++} or K^+ (H_5, H_6), but the phosphatide content appeared to be lower in cells grown at 0.6 *M* NaCl plus Mg^{++} (H_4). Although

TABLE I

Lipid content* of cells of *M. halodenitrificans*

Salt concn.	Total lipids, % dry wt.		Lipid P,† % dry wt.		Total phosphatides,‡ % dry wt.		Total non-phosphatides,§ % dry wt.	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
1.0 *M* NaCl (H_1)	12.6	9.2	0.244 (37.0)	0.237 (25.2)	5.6	5.4	7.0	3.8
0.6 *M* NaCl (H_2)	11.8	9.1	0.182 (19.6)	0.249 (18.7)	4.3	5.7	7.5	3.4
0.55 *M* NaCl (H_3)	12.6	11.8	0.234 (12.5)	0.190 (22.6)	5.4	4.3	7.2	7.5
0.6 *M* NaCl + Mg^{++} (H_4)	—	9.2	—	0.157 (18.5)	—	3.6	—	5.6
0.6 *M* NaCl + Ca^{++} (H_5)	13.0	—	0.200 (22.5)	—	4.6	—	8.4	—
0.6 *M* NaCl + K^+ (H_6)	8.7	—	0.215 (22.8)	—	5.0	—	3.7	—

*Values are given for two independent series of experiments (runs 1 and 2) made over a year apart.
†Chloroform-soluble phosphorus; values in parentheses are % of total organic P in the cells.
‡Calculated from lipid-P values, assuming a weight conversion factor of 23 (based on a mean molecular weight for the phosphates of 710).
§Values obtained by difference.

TABLE II

Analysis of total lipids of *M. halodenitrificans*

Lipid constituents*	Salt content of growth medium					
	1.0 *M* (H_1)	0.6 *M* (H_2)	0.55 *M* (H_3)	0.6 *M* + 0.01 *M* $MgSO_4$ (H_4)	0.6 *M* + 0.01 *M* $CaCl_2$ (H_5)	0.6 *M* + 0.3 *M* KCl (H_6)
P %	2.29	1.87	1.99	1.71	1.52	2.45
N %	0.95	0.77	0.59	0.79	0.50	0.76
N/P, atomic ratio	0.89	0.89	0.65	1.0	0.73	0.69
Total phosphatides, %†	52.5	42.8	45.6	39.2	34.8	56.2
Total non-phosphatides, %‡	47.5	57.2	54.4	60.8	65.2	43.8
Fatty acid, %	42.8	32.3	43.0	30.5	30.4	49.5
Neutral equivalent	267	270	272	265	269	271
Fatty acid/P, mole ratio	2.16	2.15	2.45	2.10	2.31	2.31
Unsaponifiable %	3.2	22.1	33.2	3.0	3.6	6.0
β-Hydroxybutyric acid polymer, %	28.1	16.8	13.8	51.1	—	30.0
Na %	0.82	0.63	0.66	0.51	0.50	0.58
Na/P atomic ratio	0.48	0.53	0.49	0.40	0.44	0.44
K %	0.18	0.11	0.17	0.22	0.12	0.35
K/P atomic ratio	0.06	0.06	0.07	0.10	0.06	0.11
[Na + K]/P atomic ratio	0.55	0.59	0.56	0.50	0.50	0.55

*Choline was not detected in any samples.
†Calculated from % P values assuming an average mol. wt. of the phosphatides of 710, based on an average fatty acid equiv. wt. of 270, as follows: % phosphatides = % P × 710/31.
‡Values obtained by difference.

changes in non-phosphatide content were difficult to assess, because these values were obtained indirectly, there appeared to be a trend towards higher non-phosphatide contents at the lower salt concentrations (H_2, H_3); also, there appeared to be an increase in non-phosphatide content at 0.6 *M* NaCl plus Ca^{++} (H_5), but a decrease at 0.6 *M* NaCl plus K^+ (H_6) (Table I; see also Table II). It may also be noted that whereas lipid P accounted for about 31 ± 6% of the total organic P in normal cells (H_1), it accounted for only 20 ± 2% or less in all other cells.

Analysis of the total lipids showed that salt concentration had little effect on the N/P and fatty acid/P molar ratios (Table II). The N/P ratio ranged from 0.7 to 0.9, indicating that 70–90% of phosphatide phosphorus was in

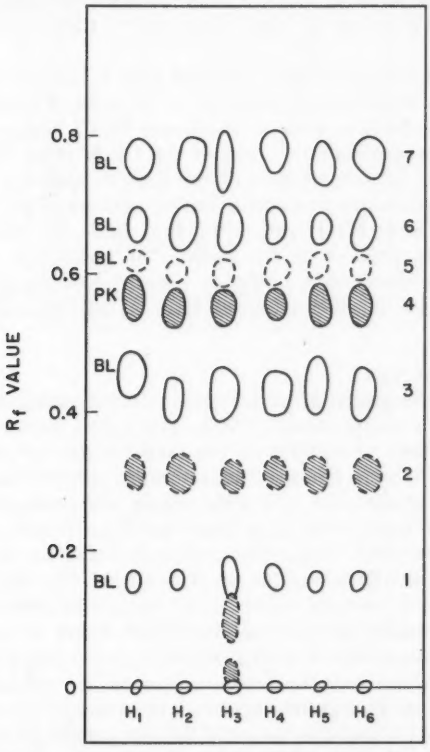

FIG. 1. Tracing of chromatogram of total lipids (7.5–8.0 μg P/spot) of *M. halodenitrificans* grown at different salt concentrations (H_1, 1.0 *M* NaCl; H_2, 0.6 *M* NaCl; H_3, 0.55 *M* NaCl; H_4, 0.6 *M* NaCl + Mg^{++}; H_5, 0.6 *M* NaCl + Ca^{++}; H_6, 0.6 *M* NaCl + 0.3 *M* KCl). The chromatogram was stained with Rhodamine 6G and viewed under ultraviolet (360 mμ) light (BL, blue fluorescence; PK, pink fluorescence). The broken lines indicate minor or trace components; the hatched spots are ninhydrin-positive. Tentative identification of components: spot 2, lysophosphatidyl ethanolamine; spot 3, phosphatidyl glycerol; spot 4, phosphatidyl ethanolamine; remaining components unidentified.

the form of nitrogen-containing phosphatides (mostly phosphatidyl ethanolamine, see Fig. 1). Absence of detectable choline showed that lecithin was not present in any of the samples. The fact that the fatty acid/P ratio was more than 2.00, the theoretical value for most phosphatides, indicates the presence in all samples of non-phosphatides containing saponifiable fatty acid ester linkages, probably glycerides. The presence of the polymer of β-hydroxybutyric acid has already been shown in this organism (18). The sum of unsaponifiable matter and polymer was usually less than total non-phosphatides, again indicating the presence of non-phosphatides with saponifiable fatty acid ester linkages. The amount of polymer in cells grown in 1.0, 0.6, and 0.55 *M* NaCl was 3.1, 1.8, and 1.7% of the dry weight respectively, indicating that the amount may decrease with salt concentration in the medium. In cells grown in 0.6 *M* NaCl plus Mg^{++}, 4.7% of the dry weight was polymer.

The amount of unsaponifiable material was 7 and 10 times greater in 0.6 and 0.55 *M* cells, respectively, than in 1.0 *M* cells. The addition of calcium, magnesium, or potassium ions to the lower NaCl concentrations decreased the amount of unsaponifiable to that of the 1.0 *M* cells. The Na/P ratio was not influenced by the salt content of the growth medium but the K/P ratio increased when potassium was added to the medium (Table II). The constant value for the (Na + K)/P ratio (0.5–0.6) indicates that 50–60% of the phosphatides were present as the sodium or potassium salts. The (Na + K)/P ratio is less than unity probably because the major component, phosphatidyl ethanolamine, is unable to bind a full equivalent of sodium or potassium.

Fatty Acid Composition

The lipids of cells grown at each of the salt concentrations contained three major fatty acid components (Table III): A C_{18}-monounsaturated acid (accounting for more than 50% of the fatty acids), a C_{16}-monounsaturated acid (about 20%), and palmitic acid (about 20%). The two unsaturated acids were most likely oleic and palmitoleic acids, respectively, since their relative retention times were very close to those of the authentic acids on Apiezon M (Table III). Trans and positional isomers of oleic and palmitoleic acids are generally separable on Apiezon M (9), but it is possible that *cis*-vaccenic (*cis*-$\Delta^{11, 12}$-octadecenoic) acid cannot be differentiated from oleic (*cis*-$\Delta^{9, 10}$-octadecenoic) acid because the double bond in the former is shifted by only two carbon atoms. For this reason, the structures assigned to the two unsaturated acids are only tentative and must be established by chemical degradative studies. Palmitoleic acid has been found in the lipids of several bacterial species (1), and has recently been reported as a major constituent of the cell envelope of *Azotobacter vinelandii* (15). Oleic acid, however, is reported to be absent in *Lactobacilli*, being replaced by *cis*-vaccenic acid (8), and in general is a minor component in most bacteria investigated (1).

Variation in the proportions of the major fatty acid components with the different salt concentrations was observed (Table III), but no significant correlation was evident. Several other fatty acids were detected in small to trace amounts in the samples. These were, in decreasing concentration:

stearic acid, a C_{13}-branched acid, a C_{17}-branched acid, heptadecanoic acid, pentadecanoic acid, and lauric acid. Traces of a C_{19} and a C_{20} acid of unidentified structure were also detected, and the concentration of these acids was consistently higher in cells grown in the presence of magnesium (Table III).

TABLE III

Fatty acid composition of lipids of *M. halodenitrificans* expressed in mole %*

Fatty acid carbon chain	Retention, relative to methyl palmitate†	Salt concentration					
		1 *M* (H_1)	0.6 *M* (H_2)	0.55 *M* (H_3)	0.6 *M* + Mg^{++} (H_4)	0.6 *M* + Ca^{++} (H_5)	0.6 *M* + K^+ (H_6)
n-C_{12}	0.16	0.1	0.1	Trace	0.1		
C_{13}-branched	0.21	1.1	1.3	Trace	0.8		
C_{14}-monoene	0.35	0.2	0.7	0.9	Trace		
n-C_{14}	0.40	0.3	0.5	0.4	0.2		
n-C_{15}	0.64	0.2	Trace	0.4	0.2		
C_{16}-monoene	0.87	24	20	21	21	23	21
n-C_{16}	1.00	21	20	17	28	26	19
C_{17}-branched	1.35	0.4	0.5	1.0	0.7		
n-C_{17}	1.58	0.4	0.2	0.9	0.4		
C_{18}-monoene	2.12	52	56	58	47	51	60
n-C_{18}	2.48	1.2	1.4	1.0	1.1		
C_{19}-unidentified	3.46	Trace	Trace	Trace	1.4		
C_{20}-unidentified	4.62	—	Trace	Trace	0.2		

*Samples of acids from H_1, H_2, H_3, and H_4 were analyzed with the Pye apparatus; H_5 and H_6 acids were analyzed with Podbielniak "Chromacon", which is not sufficiently sensitive to detect the minor components.

†At 182° on Apiezon M; relative retentions of authentic palmitoleic, oleic, and stearic acids were 0.87, 2.10, and 2.49 respectively.

Lipid Components

On chromatographing the total lipids on silicic acid impregnated paper, five major components (spots 1, 3, 4, 6, and 7) and two minor components (spots 2 and 5) were revealed by the Rhodamine 6G stain (Fig. 1). Spots 2 and 4 also gave a positive stain with ninhydrin. Spot 4, the major amino-lipid detected, was identified as phosphatidyl ethanolamine on the basis of its pink fluorescence with Rhodamine 6G (14), its R_f value [0.57; reported, 0.60 (14)], and the presence of glycerylphosphorylethanolamine [R_f in PW, 0.60; in BAW, 0.19 (11)] as the only ninhydrin-positive phosphate ester in the mild alkaline hydrolyzate of the total lipids. Spot 4 cannot be phosphatidyl serine, since the latter fluoresces blue with Rhodamine 6G (14), has a lower R_f [0.52 (14)] than phosphatidyl ethanolamine, and yields glyceryl-phosphoryl serine [R_f in PW, 0.26; in BAW, 0.12 (11)] on deacylation. The other amino-lipid detected, spot 2, was present only in traces, and its R_f value (0.30) suggests that it might be lysophosphatidyl ethanolamine.

Spot 3 was identified as phosphatidyl glycerol (2, 11) on the basis of its blue fluorescence with Rhodamine 6G (11), its R_f value [0.45; reported, 0.48 (11)], its positive stain with periodate–Schiff reagent, and the presence of diglycerophosphate [R_f in PW, 0.45; in BAW, 0.26 (11)], in the hydrolyzate. The remaining components (spots 1, 5, 6, 7) have not been identified but they are acidic since they stain blue with Rhodamine 6G. It is noteworthy that the chromatograms did not show the presence of a component corre-

sponding to lecithin.

Although there seem to be no qualitative changes in most of the lipid components under the different growth conditions, quantitative changes were evident from the size of the spots and the intensity of staining. Spot 1 is considerably larger in cells grown at 0.55 *M* salt and these cells also contain two ninhydrin-positive spots of unknown composition, although the one near the origin may be amino acids which do not move in this solvent system.

Discussion

The total lipid content of *M. halodenitrificans* grown at the various salt concentrations appeared to be fairly constant (11 $\pm$ 2% of dry weight), but the proportions of phosphatide, unsaponifiable material, and β-hydroxybutyrate polymer were influenced by the salt concentration of the growth medium. Thus, the proportion of phosphatide in the total lipids decreased with decreasing sodium chloride concentration even in the presence of added magnesium or calcium ion; the proportion of unsaponifiable matter increased with decreasing salt concentration, but was restored to normal in the presence of added magnesium or calcium ion; and the proportion of polymer decreased at low salt concentrations, but was much higher than normal in the presence of magnesium ion (see Table II).

The most striking change was the increase in the unsaponifiable matter in cells grown at low salt concentrations, and it would be worthwhile determining whether this material was associated with the cell membranes or with the cytoplasm, and also whether the increase resulted from interference with normal lipid metabolism. Since sterols are generally absent in bacteria (1, 13), the unsaponifiable matter found in *M. halodenitrificans* may perhaps be long chain alcohols (derived from waxes), long chain ketones, or long chain aldehydes (derived from plasmalogens). The last possibility, however, is virtually eliminated by the fact that the total lipids were found to contain only traces of aldehydes.

Since the β-hydroxybutyrate polymer is probably a reserve cytoplasmic material, it may be that the decreased amount of polymer in cells grown at low salt concentrations results from the unfavorable growth conditions. This effect is being investigated further.

The phosphatide composition of *M. halodenitrificans* is unusual in several respects. Lecithin, the major phosphatide component in higher plants and animals, is completely absent. The absence or low content of lecithin in other bacteria has however been noted previously (1), and recently the lipids of another halophile, *Halobacterium cutirubrum*, have also been found deficient in lecithin (17). The other "classical" phosphatide, phosphatidyl ethanolamine, is one of the major components in *M. halodenitrificans* (see Fig. 1), but has been reported present only in relatively few bacteria (1), and has even been considered to be absent in bacteria generally (13). *M. halodenitrificans* also contains at least five acidic phosphatides, one of which (spot 3, Fig. 1) is most likely phosphatidyl glycerol, previously found in high concentration only in plants (2, 11). The R_f values of some of the other acidic phosphatides suggest that they may be phosphatidyl inositol [spot 1, Fig. 1; see (11, 14)], polyglycerolphosphatides [spots 5 and 6, Fig. 1; see

(14)], and phosphatidic acid [(spot 7, Fig. 1; see (11)]. It is of interest that the lipids of protoplast membranes of *Micrococcus lysodeikticus* have recently been found to consist almost entirely of a complex phosphatidic acid (7). The acidic phosphatides in *M. halodenitrificans* may possibly be associated with the cell membrane, and these components will merit further investigation.

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STUDIES ON THE INTERACTION OF PLANTS AND FREE-LIVING NITROGEN-FIXING MICROORGANISMS

I. OCCURRENCE OF AZOTOBACTER IN THE RHIZOSPHERE OF CROP PLANTS[1]

H. KATZNELSON AND E. STRZELCZYK[2]

Abstract

The occurrence of *Azotobacter* in the rhizosphere of 17 crop plants grown in different types of soil in the field and in the greenhouse has been studied as part of a program of work on the interaction of plants and free-living nitrogen-fixing microorganisms. *Azotobacter* counts were very low in both rhizosphere and non-rhizosphere soil from the field; they varied with the type and age of the plant and with the type of soil. The organism was usually absent from acidic and poor soils. In the greenhouse experiments, soil counts of these bacteria were found to be somewhat higher than in the corresponding soil in the field (Granby sandy loam). The root effect was greater with both radish and wheat in the poor soil (Upland sand) than in the fertile Granby soil. Onion exerted no obvious influence on the *Azotobacter* population in either soil. However, even under optimum conditions of plant growth, numbers of these organisms were very low in comparison with those of other bacteria normally present in soil and in the root zone.

Introduction

It is well established that soil in the immediate vicinity of plant roots (rhizosphere) contains larger numbers of organisms than soil more distant from the roots. There is evidence also that the rhizosphere microflora differs qualitatively from that in non-rhizosphere soil (11, 12, 13, 17, 19, 20, 29). Of the many types of organisms found on plant roots, the nitrogen-fixing forms are of obvious interest. The importance of the root nodule bacteria of leguminous plants is well established today and there is little argument about their value to these plants. The situation with respect to non-symbiotic nitrogen-fixing organisms is somewhat different. Controversy over their actual importance in soil, or more directly to the plant, is still going on even though the weight of evidence in the U.S.S.R. appears to favor the use of *Azotobacter* as a seed treatment (4), not only because of its nitrogen-fixing ability but also because of its growth factor and auxin-synthesizing capacity (2, 7, 26). Under natural conditions, however, and especially in the temperate zones, *Azotobacter* does not usually occur abundantly in soil or in the root zone (8, 12). *Clostridium* species appear to be more abundant (9), and other types reported more recently to be able to fix small amounts of atmospheric nitrogen may also be of some importance (5, 22, 25, 39, 42). It is the purpose of this series of papers to examine the interaction of plants and free-living nitrogen-fixing soil microorganisms as regards numbers and types occurring in the root zone of common crop plants and with a view to determining their importance to the plant.

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Historical

Azotobacter is a widely distributed soil organism commonly regarded as being of some importance in most arable soils as a result of its nitrogen-fixing ability (7, 26, 39) and its production of growth factors (2). The development or survival of *Azotobacter* occurring naturally in or introduced into soil depends on the presence of an available energy source; minerals such as calcium, phosphorus, potassium, iron, and molybdenum; proper soil aeration; suitable moisture; and favorable soil reaction (8). Studies on the influence of plants on this organism were made as early as 1908 by Beijerinck (1), who found greater numbers close to the roots of leguminous plants than in the rhizosphere of non-leguminous plants. Kostychev *et al.* (14) also noted an accumulation of *Azotobacter* in the vicinity of roots. Starkey (33), however, could obtain no evidence of a selective action of roots of legumes or non-legumes on these bacteria. Poschenrieder (24) found *Azotobacter* to be very numerous in the rhizosphere of the Cruciferae. In an investigation of the distribution of this nitrogen-fixing bacterium in the root system of plants belonging to 31 families Rokitzkaya (27) noted its presence in all but three (Pinaceae, Betulasceae, and Fagaceae). Sheloumova and Menkina (30) observed the development of *Azotobacter* in the rhizosphere of corn and buckwheat throughout the early stage of growth. According to Krassilnikov (15) these organisms were absent from the rhizosphere of wheat and developed only weakly in that of corn. The same author divided crop plants into groups according to their effect on *Azotobacter*. Leguminous plants were found to enhance the *Azotobacter* population; wheat, corn, flax, and cotton had an inhibitory effect, while oats, barley, potatoes, and sunflower had no influence. Katznelson (9) recorded only a slight stimulation of *Azotobacter* by mangels. Sidorenko (31) reported that oats, barley, sudan grass, sunflower, and soybeans stimulated the development of *Azotobacter* inoculated on seed; wheat depressed it; and corn, soybean, and alfalfa caused fluctuations according to the stage of plant growth. Katznelson *et al.* (13) could not demonstrate a rhizosphere effect on these organisms with wheat, barley, and soybeans. In recent studies carried out in Poland, Ziemiecka and Maliszewska (45) and Strzelczyk (36) noted a "rhizosphere effect" of certain crop plants on *Azotobacter*. This effect differed according to the kind and age of the plant. Stille (34) found a distinct difference in the rhizosphere effect on this organism according to the type of soil in which the plants were grown, the effect being greater in a poor, sandy soil than in a heavier one.

Surprisingly little attention has been paid to other nitrogen-fixing organisms in the rhizosphere. Of these, the anaerobic types such as the clostridia may be very important in view of the fact that they are more abundant in soil and are less sensitive to a wide variety of environmental factors (moisture, temperature, reaction) than *Azotobacter* (41). Katznelson (9) observed a rhizosphere effect of mangels on gas-producing anaerobes, very closely related to, if not identical with, *Clostridium pasteurianum*. Krassilnikov (16) and Vozniakovskaya (40) noted the accumulation of *Clostridium pasteurianum* in the rhizosphere of wheat and corn. Recently, Strzelczyk (36) also reported a rhizosphere effect of certain crop plants on *Clostridium*. Nitrogen fixation has been reported to occur with photosynthetic bacteria such as *Rhodo-*

spirillum rubrum, *Chlorobium* sp., *Chromatium* sp., and in some blue-green algae (*Nostoc muscorum* and *Anabaena cylindrica*) (22). *Aerobacter aerogenes*, *Methanobacterium omelianskii*, soil yeasts such as *Saccharomyces* sp. and *Rhodotorula* sp. (22), *Achromobacter* spp. (25), *Pseudomonas*, and *Nocardia* (39, 42) have also been reported to fix nitrogen. *Pseudomonas* species abound in the rhizosphere and rhizoplane of plants, comprising, in some cases, between 40 and 50% of the bacterial population (10, 28). There are often well over 1 billion viable bacteria in 1 g rhizosphere soil; thus there may be 400 or 500 million pseudomonads. If some of these are nitrogen-fixers a significant amount of nitrogen may be made available to the plant.

Very little has been done in Canada on the influence of plant roots on these free-living nitrogen-fixing bacteria (9, 13). It was considered important, therefore, to initiate an investigation on the numbers and types of these organisms in the rhizosphere of common crop plants grown in this country and to determine their influence on plant growth under natural and greenhouse conditions as affected also by soil and seed inoculation. The results of these studies will be reported in this series of papers.

Materials and Methods

Samples of roots and of adjacent root-free soil were taken from four locations in a field. Each sample consisted of 10 to 12 plants and of 5 to 10 scoops of soil which were thoroughly mixed. Rhizosphere soil was obtained by immersing the roots in sterile water blanks, shaking the containers for 5 minutes, and removing the roots. Dilutions were then prepared from this suspension as well as from a water suspension of the root-free soil sample. Numbers of *Azotobacter* were determined by spreading 1 ml of each dilution (in quintupli-

TABLE I

Description of soils

Type of soil	Organic matter content (%)	Easily soluble P and replaceable K (lb/acre)	Fertilizers used (lb/acre)	pH
Allandale sandy loam	—	—	Superphosphate, 20–150	7.0
Grenville sandy loam	6.6	P, 72 K, 174	Nitrate of soda, 100 Superphosphate, 300 Muriate of potash, 75	7.2
Granby sandy loam	3.8	P, 129 K, 60	N–P–K (4–24–12), 300	7.0
Manotick sandy loam	4.1	P, 81 K, 123	Superphosphate, 20–150	
Matilda loam	—	—	Superphosphate, 20–150	7.0
North Gower clay	7.1	P, 373 K, 269	Superphosphate, 20–150	7.1
Rideau clay	5.7	P, 231 K, 318	N–P–K (4–24–12), 180	6.0
Kars gravelly sandy loam	2.9	P, 60–70 K, 104	—	6.6
Upland sand	1.2	—	—	5.0

cate) uniformly over the surface of nitrogen-free sucrose agar plates (K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.2 g; $MnSO_4$, $FeSO_4$, $NaMoO_4$, trace; $CaCO_3$, 5.0 g; sucrose, 10.0 g; agar, 15.0 g; distilled water, 1000 ml, sterilized by autoclaving for 15 minutes at 15 lb). The plates were dried at 35 to 38° C for a short time and colonies were counted after 2 to 3 days' incubation at 28° C.

The soils used represent a cross section of types found at the Central Experimental Farm in Ottawa. As may be seen in Table I, they range from sands to clays and from 3 to 7% organic matter. The fertilizer treatment varied with the soil type and was usually high in phosphorus.

In order to examine the influence on the *Azotobacter* population of specific crops and the effect of soils differing markedly in fertility, the following greenhouse experiment was conducted. Wheat, radish, and onion were grown in 9-in. pots in two different soils: Granby sandy loam (pH 7.0; organic matter, 5%) and Upland sand (pH 5.0; organic matter, 1.2%). In view of the acidic nature of the latter soil and its complete lack of *Azotobacter*, 0.5% $CaCO_3$ and an *Azotobacter* inoculum consisting of 10% Granby sandy loam were thoroughly worked into it prior to planting. *Azotobacter* counts were made 21, 70, and 100 days after seeding.

Results

Data on the occurrence of *Azotobacter* in the rhizosphere of 15 different crop plants at two stages of growth and in root-free (control) soil are presented in Table II. A total of 248 samples was analyzed. *Azotobacter* counts were extremely low in all samples as compared with bacterial numbers, which were in the millions (37). *Azotobacter chroococcum* was the only species isolated in these tests. Despite the low counts certain trends are worthy of comment. The incidence of *Azotobacter* appears to be influenced by the type and age of the plant and by the nature of the soil. The greatest number was found in the rhizosphere of buckwheat (rhizosphere:soil ratio of 17.8), whereas tobacco, mustard, and flax had little effect. Rye, oats, wheat, corn, and peas showed a greater rhizosphere effect in the seedling stage than in the flowering stage. In several instances (field peas, flax) numbers actually seemed to be depressed in the rhizosphere at the latter sampling period. On the other hand, *Azotobacter* found the most favorable conditions on buckwheat roots at the flowering stage.

The soils apart from the roots were very low in *Azotobacter* as reported also by Lochhead and Thexton (18). However, the organism occurred in all the soils studied except Rideau clay and Upland sand, varying in number with soil type, from 2 to 140 per g soil. It was absent from the rhizosphere of safflower, poppy, mustard, flax, and buckwheat grown in the Rideau clay.

The results of the greenhouse tests are presented in Table III. Again it may be noted that the incidence of *Azotobacter* in the rhizosphere is affected by the type of plant used and by its age. Onion exerted little, if any, significant effect. Radish was ineffective when grown in the fertile soil (Granby sandy loam) but stimulated *Azotobacter* growth in the poor soil (Upland sand). This favorable effect increased with time until at 100 days a rhizosphere:soil ratio (R:C) of 13.4 was obtained. Wheat roots appeared to be more favorable for this organism in both soils than either of the other two

TABLE II

Occurrence of *Azotobacter* in rhizosphere and non-rhizosphere soils

Plant	Stage of growth	Type of soil	Numbers of *Azotobacter** (av of 4 samples)			Remarks
			Rhizosphere soil	Control soil	R:C†	
Rye	Seedling	North Gower	84	13	6.4	Spring crop
	Flowering	clay loam	16	10	1.6	
Oats	Seedling	North Gower	79	8	9.8	Winter crop
	Flowering	clay loam	28	15	2.1	1959–60
	Seedling	Matilda loam	49	24	2.0	Spring crop
	Seedling	North Gower clay loam	80	11	7.2	Winter crop 1960–61
Wheat	Seedling	North Gower	75	9	8.3	Winter crop
	Flowering	clay loam	28	13	2.1	1959–60
	Seedling	Matilda loam	57	24	2.3	Spring crop
	Seedling	North Gower clay loam	77	10	7.7	Winter crop 1960–61
Barley	Seedling	North Gower	70	13	5.3	Winter crop
	Flowering	clay loam	70	18	3.8	1959–60
	Seedling	Matilda loam	114	16	7.1	Spring crop
	Seedling	North Gower clay loam	51	13	3.9	Winter crop 1960–61
Corn	Seedling	Grenville sandy	153	27	5.6	Spring crop
	Flowering	loam	92	49	1.8	
Flax	Seedling	Allandale	211	110	1.9	
	Flowering	sandy loam	35	90	0.3	
	Flowering		75	80	0.9	Spring crop
Field peas	Seedling	Manotick sandy	88	17	5.1	
	Flowering	loam	7	29	0.2	Spring crop
	Flowering	—	0	2	—	

TABLE II (*Concluded*)

Occurrence of *Azotobacter* in rhizosphere and non-rhizosphere soils

Plant	Stage of growth	Type of soil	Numbers of *Azotobacter** (av. of 4 samples)			Remarks
			Rhizosphere soil	Control soil	R:C†	
Field beans	Seedling	Allandale sandy loam	94	100	0.9	Spring crop
	Flowering		174	139	1.2	
	Seedling	Kars gravelly sandy loam	28	10	2.8	
	Flowering		80	21	3.8	
Soybeans	Seedling	Granby sandy loam	111	32	3.4	Spring crop
	Flowering		141	54	2.6	
Rape	Flowering	—	6	2	3.0	
Buckwheat	Seedling	Granby sandy loam	186	29	6.4	Greenhouse plants
	Flowering		410	23	17.8	
Mustard	Seedling		32	27	1.2	
	Flowering		53	38	1.4	
Tobacco	Seedling		42	27	1.5	
	Flowering		0	28	—	
Safflower	Seedling		87	29	3.0	
Poppy	Seedling		218	22	9.9	

*Numbers per g oven-dry, root-free or rhizosphere soil.

†$R:C = \frac{\text{Numbers in rhizosphere soil}}{\text{Numbers in control soil}}$.

TABLE III

The influence of age, kind of plant, and type of soil on numbers of *Azotobacter* in rhizosphere and control soils*

Type of soil	Age of plant (days)	Control soil	Rhizosphere soil					
			Radish	R:C†	Onion	R:C	Wheat	R:C
Granby sandy loam	21	805	1116	1.3	958	1.2	3492	4.3
	70	313	469	1.5	321	1.0	6397	20.4
	100	404	370	0.9	280	0.6	—	—
Upland sand	21	48	169	3.5	86	1.8	2935	61.1
	70	30	176	5.8	20	0.6	4371	145.7
	100	31	414	13.4	44	1.4	—	—

*Numbers per g oven-dry, root-free or rhizosphere soil.

†$R:C = \frac{\text{Numbers in rhizosphere soil}}{\text{Numbers in control soil}}$.

crops. This was especially noticeable in the Upland sand at both 21 and 70 days, at which time the R:C values were 61 and 146, respectively. The counts obtained in the rhizosphere soil of wheat grown in the greenhouse are considerably higher than those for wheat grown in the field, suggesting the operation of other factors (in addition to soil type), such as moisture and temperature, in suppressing *Azotobacter* development in the field. However, even under the favorable conditions supplied in the greenhouse, the counts were very much lower than those of many other bacteria normally occurring in both soil and rhizosphere.

Discussion

The evidence presented indicates clearly that under field conditions the root zone is not a favorable habitat for *Azotobacter* occurring naturally in soil. Although there are usually larger numbers in the rhizosphere than in root-free soil, the counts are so low as to suggest its suppression. This would appear to be the case also with greenhouse-grown plants even though the rhizosphere counts in certain cases (wheat) are considerably higher than those in the root-free soil. The inhibition of *Azotobacter* may be due to a number of agencies operating at the root–soil interface. Extracts of certain roots are known to inhibit *Azotobacter* (3, 21). However, this is probably not the major reason for its poor growth on the roots of most crop plants. A much more likely explanation is the intense antagonism which may be exerted by the dense microbial population in the rhizosphere resulting in competition for food, oxygen, and space and (or) the production of metabolites which may inhibit *Azotobacter* directly, or indirectly by altering the pH, oxidation–reduction potential, etc. (12, 32). These factors are undoubtedly operative in the root-free soil as well (8). The rapid growth and metabolism of rhizosphere bacteria as reported by Katznelson and his co-workers (10, 44), Rovira (29), and Macura (20) may prevent *Azotobacter*, a relatively slow grower, from becoming established in this zone. Fedorov and Tepper (6) also found that a number of bacteria were capable of suppressing *Azotobacter* in the rhizosphere, and Stille (34, 35) is of the opinion that *Pseudomonas fluorescens* is the chief antagonist. Certainly pseudomonads are among the most numerous and rapidly growing of the bacteria in the rhizosphere (10, 28) and may exert a profound effect on *Azotobacter*. The ability of various soil organisms to inhibit *Azotobacter* is well recognized (23, 38, 43).

This phenomenon of antibiosis will be considered in greater detail in the next paper of this series.

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SUBMERGED CITRIC ACID FERMENTATION OF SUGAR BEET MOLASSES: EFFECT OF PRESSURE AND RECIRCULATION OF OXYGEN[1, 2]

D. S. CLARK AND C. P. LENTZ

Abstract

Recirculation of oxygen during submerged citric acid fermentation of ferrocyanide-treated beet molasses by *Aspergillus niger* was successful. Results obtained in 36-liter glass tower fermenters showed that neither rate of production nor total yield of citric acid was affected. No treatment of the recirculated gas other than carbon dioxide removal was required. At both low and high oxygen flow rates, increased oxygen pressure (up to 1.7 atm) markedly increased initial rate of production and total yield of citric acid. Oxygen pressure above 1.7 atm decreased citric acid yield. Within the limits tested, the effect of oxygen pressure was independent of total pressure.

Introduction

Recent work on submerged citric acid fermentation carried out in small (2.5-l.) and large (36-l.) tower fermenters has shown that ferrocyanide-treated beet molasses can be fermented rapidly by *A. niger* to produce high yields of citric acid (3, 4, 6, 7). However, one disadvantage of the method was the high volume flow of oxygen necessary during most of the fermentation, even though only a small fraction of this gas was consumed. Since the critical nature of the fermentation made it difficult to predict the effect of oxygen-saving methods on the fermentation, a study was made of the effects of oxygen recirculation, oxygen partial pressure, and total pressure on the rate of production and on yield of citric acid. Rates of oxygen consumption and carbon dioxide production during fermentation were also measured in recirculation experiments.

Methods

All tests were made with either Chatham 1957 or Chatham 1958 beet molasses* using mainly the techniques and equipment described previously (6, 7). Briefly, the molasses was diluted with tap water to 12% sugar concentration and adjusted to pH 5.5 when used for seed cultures, and to pH 6.0 when used for fermentation. After sterilization, the mash was treated, while still hot, with potassium ferrocyanide (0.4 g/l., Chatham 1957 molasses; 0.7 g/l., Chatham 1958 molasses). In seed culture preparation, 1500-ml quantities of mash in 6-l. flasks were readjusted to pH 6.0, inoculated with 10^6 spores of *A. niger* N.R.C. A-1-233, and incubated at 29° C for 20–24 hours on a rotary shaker. The pellets that developed were counted visually, and appropriate quantities of the suspensions were used to inoculate mash in

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the fermenters. Mash used for fermentation was readjusted to pH 6.5 (Chatham 1958 molasses) or to pH 7.0 (Chatham 1957 molasses) and measured (20 l.) into a sterilized 6-in. diameter, 36-l. capacity, pyrex tower fermenter (described in earlier papers (5, 6)). The temperature in the tower was controlled at 31° C, and corn oil was used during the first 48 hours of the fermentation to assist in foam breaking. The mash was inoculated with 4 million pellets, sparged with air for the first 24 hours of fermentation, and with air, oxygen, or mixtures of air and oxygen as desired for the remaining time. Two fermenters were run simultaneously, one serving as a control.

In recirculation experiments, air or oxygen supplied to the fermentation was continuously recirculated through the fermenter in an essentially closed system. In this system, the gas leaving the outlet at the top of the fermenter was pumped by an oilless, rubber-diaphragm pump* through 1/4-in. diameter copper tubing to a carbon dioxide scrubber and a calibrated rotameter (connected in series) back to the inlet at the bottom of the fermenter. The gas flow rate through the fermenter during recirculation was 7000 cc/minute. A spirometer–kymograph assembly, connected into the line between the diaphragm pump and the top of the fermenter, continuously supplied and accurately recorded the amount of oxygen required to replace that consumed during fermentation. Carbon dioxide was absorbed from the system by bubbling the recirculating gas through a 2.5-ft column of a 20% sodium hydroxide solution. The closed system was tested for leaks before each experiment by adding freon-12 under a small pressure and checking all connections with a freon leak detector. When the air was replaced by oxygen, the system was flushed with oxygen until all the nitrogen was removed. In control fermentations, air and oxygen were not recirculated; otherwise, conditions were the same as those in the tests.

The effect of oxygen pressure on the fermentation was studied by using mixtures of air and oxygen for oxygen partial pressures of less than 1 atm, and by using pure oxygen for pressures of 1 atm and greater. At 2 oxygen pressures (1 and 1.7 atm), the effect was studied over a range of gas flow rates. The effect of total pressure was assessed by using different levels of total pressure but the same oxygen partial pressure. The maximum total pressure used was limited to about 2 atm by the strength of the glass equipment. Increased total pressures in the fermenter were maintained automatically by a pneumatic pressure controller attached to the gas outlet at the top of the fermenter (accuracy ± 0.02 atm). To obtain mixtures of air and oxygen, the two gases were metered through separate rotameters and mixed immediately before entering the fermenter (accuracy of mixing, $\pm 2\%$ of oxygen partial pressure desired). In control fermentations, the oxygen partial pressure (from the 24-hour period onward) and the total pressure in the fermenter were always 1 atm; all other experimental conditions were the same as those used in the tests. In pressure experiments, the gases used were not recirculated.

Citric acid concentration in the fermenting mash in all experiments and the carbon dioxide concentration in the scrubber liquid during recirculation tests were determined at regular intervals during the time the fermentations were

*Air-Shields, Inc., Hatboro, Penn.

allowed to run (140 hours). Citric acid was determined by the method described by Marier and Boulet (2). Carbon dioxide was determined by titrating the unused sodium hydroxide in the scrubber liquid with hydrochloric acid to the phenolphthalein end point after precipitating the carbonate present by addition of excess barium chloride. Sucrose in the mash, measured only at the beginning of the fermentation, was determined by a procedure outlined by Dubois *et al.* (1). Acid yields in recirculation tests were corrected for volume loss in the fermenter (about 2% after 140 hours) resulting from moisture absorption by sodium hydroxide in the scrubber. In all other tests acid yields were corrected for evaporation loss (about 8% by volume after 140 hours). Carbon dioxide yields were corrected for water absorption by sodium hydroxide solution in the scrubber.

Results and Discussion

Recirculation of oxygen in an essentially closed system was used successfully in the fermentation. There was negligible difference between yields in fermentations with recirculation and yields in control fermentations (average yield in seven experiments, 9.2%). Recirculation had no noticeable effect on pellet formation or on rate of acid development. Apparently no volatile or gaseous substances deleterious to acid production were produced during fermentation, or if so they were removed along with carbon dioxide by the scrubber liquid.

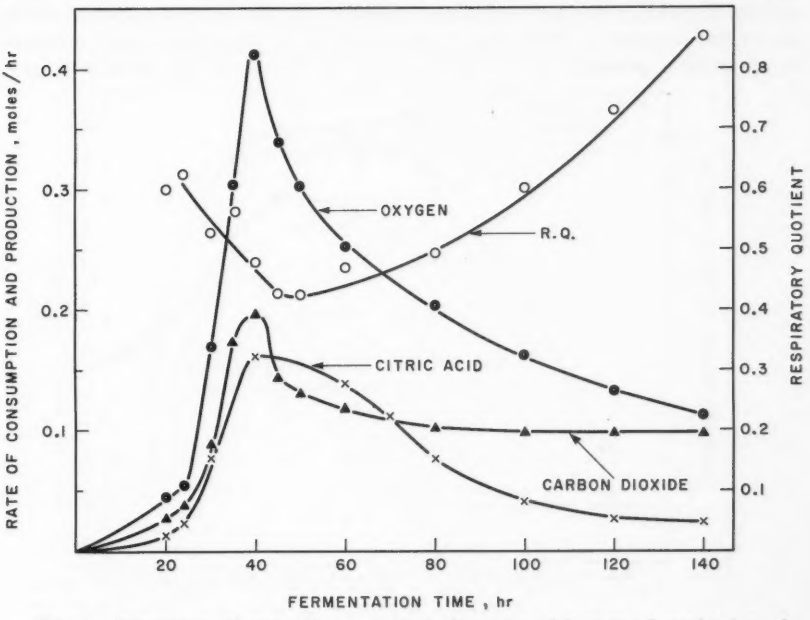

FIG. 1. Comparison of rate of consumption of oxygen with rates of production of citric acid and carbon dioxide (Chatham 1957 molasses; air used for first 24 hours; average of four fermentations).

Oxygen uptake and carbon dioxide production measurements made during recirculation experiments permitted comparison of the rate of oxygen consumption with the rate of carbon dioxide production as well as the determination of the respiratory quotients for *A. niger* during these relatively large-scale fermentations. Figure 1 shows the average results of four fermentations in which air (for the first 24 hours of fermentation) as well as oxygen was recirculated. The rates of oxygen consumption and carbon dioxide and citric acid production increased sharply at the 24-hour period of fermentation, when oxygen replaced air, and reached a maximum at about the 40-hour period. The maximum rates of consumption of oxygen and production of carbon dioxide were about double the average rates but continued for only a short period of time. Although the maximum rate of citric acid production continued for a longer period of time, citric acid production appeared to be related to oxygen consumption. The steady production of carbon dioxide during the last half of the fermentation when the rate of oxygen consumption was declining resulted in a rise in the respiratory quotient from a low of 0.42 at the 50-hour period to a high of 0.85 at the 140-hour period. In these four fermentations, an average of 25.6 moles of oxygen was consumed and 14.1 moles of carbon dioxide and 9.5 moles of citric acid produced in 140 hours. The average conversion of sucrose to citric acid was 76% by weight.

Results of pressure experiments showed that rate of citric acid production and total yield in the fermentation depended on the oxygen pressure in the gas in the fermenter. Maximum yield and rate of production were obtained at an oxygen pressure of 1.7 atm; higher or lower oxygen pressures gave

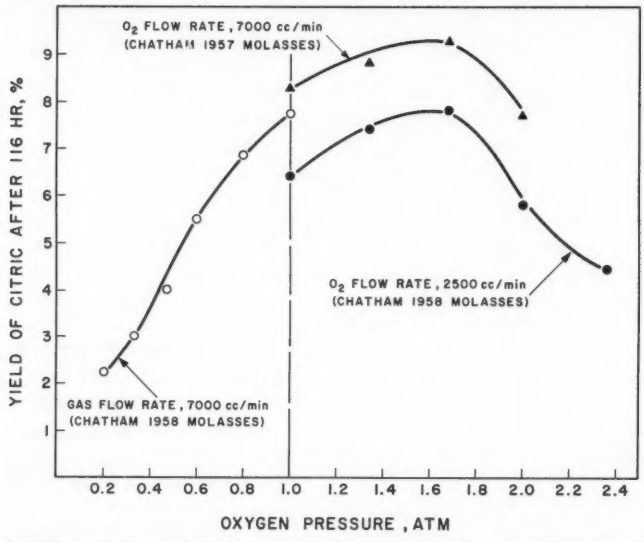

FIG. 2. Effect of oxygen partial pressure on yield of citric acid. (Total pressure was 1 atm where oxygen partial pressure was less than 1 atm, otherwise the oxygen pressure indicated was the total pressure.)

lower rates and yields (Fig. 2). The beneficial effect of oxygen pressure greater than 1 atm appeared to be independent of crop year of molasses, although the effect was relatively greater for the lower of the two oxygen flow rates used. The consistent difference (approximately 8%) between yields with Chatham 1957 molasses and yields with Chatham 1958 molasses, at 1 atm oxygen pressure and at a high oxygen flow rate, was presumably related to the fermentability of the two molasses. Chatham 1958 molasses was used when the supply of Chatham 1957 molasses was exhausted.

Results of experiments with mixtures of air and oxygen showed that, within the limits tested, the effect of oxygen pressure was independent of total pressure; differences between yields obtained at different total pressures (the oxygen pressure remaining the same) were negligible. Thus the required oxygen pressure could be obtained by increasing either the oxygen concentration in the gas supply or the total pressure in the fermenter.

The beneficial effect of increased oxygen pressure was more pronounced at low than at high oxygen flow rates (Fig. 3). This resulted in high acid yields at lower gas flow rates than could be successfully used without increased oxygen pressure. Below a flow rate of 2500 cc/minute, the pellets packed at the bottom of the fermenter and yields dropped markedly at all pressures. These results indicate that, within limits, increased oxygen pressure will permit the use of lower oxygen flow rates.

Comparison of rates of citric acid production (rather than final yield)

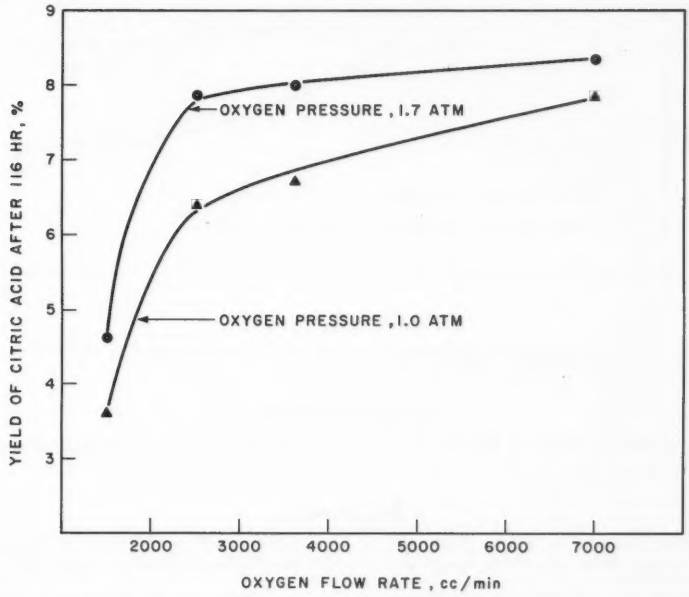

FIG. 3. Effect of oxygen flow rate on yield of citric acid at two oxygen pressures (Chatham 1958 molasses).

at different oxygen pressures during the fermentation (Fig. 4) showed that the increased yield with increased pressure was the result of a faster initial rate of citric acid production. Apparently rate of oxygenation is only limiting during the first half of the fermentation, when oxygen consumption is greatest; rate of citric acid production during the last half of the fermentation must, therefore, be determined by factors other than oxygen supply. The results suggest that increased oxygen pressure need only be used during the first half of the fermentation to achieve maximum yields.

Oxygen recirculation, and flexibility in obtaining suitable oxygenation, should greatly increase the economic attractiveness of this fermentation.

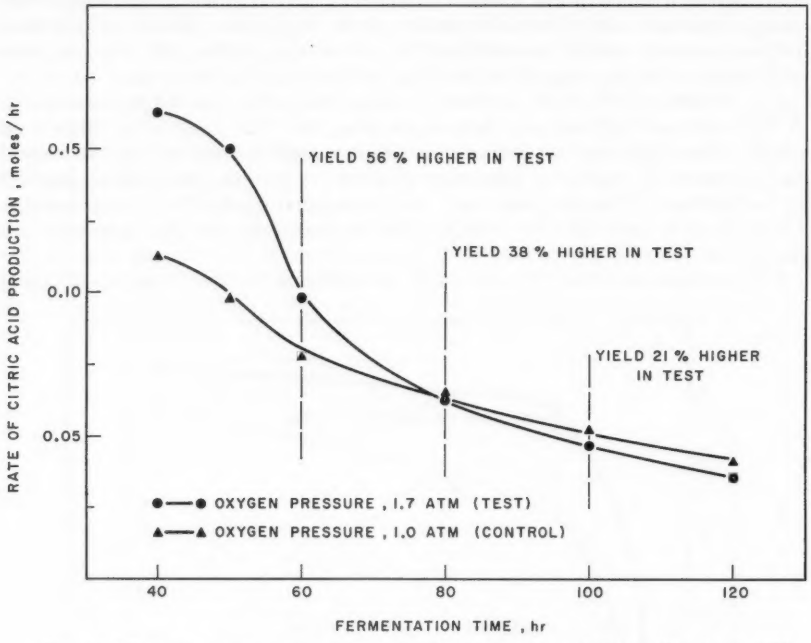

FIG. 4. Effect of oxygen pressure on rate of citric acid production (Chatham 1958 molasses; oxygen flow rate, 2500 cc/minute).

Acknowledgment

The authors wish to thank N. U. Cholette for technical assistance during this investigation.

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LYSOGENY IN RHIZOBIUM TRIFOLII[1]

I. TAKAHASHI AND C. QUADLING[2]

Abstract

A phage-like particle (phage i) found in filtrates of *Rhizobium trifolii* (strain RT 9) did not produce plaques but induced development of two different phages in a presumed defective lysogenic strain (RT 10). These induced phages (phages 9 and 10) differed in their host ranges and produced plaques on strains RT 9 and RT 10, respectively. Phages 9 and 10 were closely related serologically and were distinct from phage i. Although ultraviolet irradiation resulted in the lysis of RT 10, no free phage was detectable in such lysates. Phage i particles had morphological, chemical, and physical properties similar to those of functional *Rhizobium* phages but did not produce plaques on any of 12 *R. trifolii* strains tested. Development of phage i could be induced with ultraviolet irradiation or by treatment with mitomycin C.

Introduction

It is well known that many bacterial strains are lysogenic, that is, they have an inherited potentiality for producing bacteriophage either spontaneously or under appropriate environmental conditions. The lysogenic condition has been the subject of recent reviews (1, 2, 13).

The existence of a lysogenic strain of *Rhizobium trifolii* was reported by Marshall (14). He observed plaques on an indicator strain (SU 297) when it was inoculated with his lysogenic strain (SU 298) and also observed that his lysogenic strain was resistant to purified phage. Our observations with these strains confirm his findings but show that the circumstances leading to the production of functional phage by these two strains were more complex than previously reported. This paper describes a phage-like particle which is present in culture filtrates of Marshall's indicator strain. Infection with this particle is a prerequisite for the production of functional phage by Marshall's reported lysogenic strain, and no plaque-forming particle could be detected in cultures of this so-called lysogenic strain in the absence of such prior infection.

Materials and Methods

Bacterial Strains and Cultural Methods

Two strains of *Rhizobium trifolii* which form the subject matter of this paper were obtained from Marshall via the School of Agriculture, University of Sydney, Australia. Our RT 9 was isolated from a transfer of SU 297 (indicator strain) by plating and picking a single colony. RT 10 was similarly derived from SU 298 (lysogenic strain). Other *R. trifolii* strains used were from the culture collection of this laboratory. Cultures were preserved in liquid medium Y (5) containing 15% glycerol and stored at −35° C (18). Active cultures in late logarithmic phase were obtained by inoculating 0.2 ml

[1]Manuscript received October 31, 1960.
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frozen culture into 50 ml liquid medium in a 250-ml Erlenmeyer flask and incubating it at 25° C on a rotary shaker for 24 hours. The term "broth" in this paper refers to liquid medium Y; "agar medium" refers to medium Y solidified with agar.

Numbers of viable bacteria were determined by a pour-plate technique using medium Y containing 1% agar. Colonies were counted after 4 days. Since dense broth cultures of RT 10 sometimes showed spontaneous clumping or failure of separation of bacteria, viable counts must be considered as minimal estimates of bacterial numbers. RT 9 usually had a diffuse mode of growth in liquid medium. These organisms had a mean generation time of about 3.5 hours in broth.

Phage Techniques

Numbers of plaque-forming particles were determined by the soft agar layer method described by Potter and Nelson (17). Medium Y was used as diluent and for adsorption mixtures. The same medium with the addition of 1% agar was used for the basal layer of assay plates. After 15 minutes' preincubation at room temperature and addition of 0.5% agar the mixtures of phage and indicator bacteria were overlaid. Plaques were counted after 24 hours' incubation at room temperature (approx. 25° C).

In general, the techniques used were those of Adams (1). Filtrates free of bacteria were obtained by means of appropriate sintered glass filters or by means of membrane filters. When necessary, bacteria were killed by addition of sufficient chloroform to give a saturated solution (1) which was later removed by aeration. This treatment did not affect phage titers. Ultraviolet irradiation of cultures was carried out at a distance of 20 cm from a "Mineralight" source (Ultraviolet Products Inc., South Pasadena, California) equipped with a 2537 Å filter.

Electron Microscopy

A pseudoreplica technique (23) was used for making electron micrographs of phages. Cell lysis was completed in broth and a 2% agar layer flooded with the suspension. On drying, this agar was used to form collodion pseudoreplicas.

Results

Initial Observations

When strains RT 9 and RT 10 were cross-streaked on the surface of agar medium and incubated, an area of visible lysis developed at the junction of the streaks. Similarly, when these two strains were grown together in mixed liquid culture, a phage active on RT 9 could be isolated from sterile filtrates or from chloroform-treated culture supernatants. As might be expected, when culture dilutions of RT 10 were plated, there was a close correspondence between the number of colonies which appeared on agar medium and the number of plaques formed on soft agar layer plates inoculated with RT 9 as indicator. Furthermore, purified phage active on RT 9 was found to be unable to form plaques on RT 10, although the phage was adsorbed by RT 10 bacteria. These observations are in accordance with those of Marshall (14); they suggest that RT 10 is lysogenic for a phage which forms plaques on RT 9 and also that RT 10 cells burst to liberate this phage with measurable fre-

quency. Surprisingly, no such free phage could be demonstrated in culture filtrates of RT 10 grown alone or in chloroform-treated RT 10 cultures. However, both treatment with ultraviolet irradiation (95% killing dose) and exposure to mitomycin C,[3] which mimics ultraviolet irradiation and induces prophage development in *Escherichia coli* (15), resulted in the eventual lysis of RT 10 cultures. Even under these conditions, no free phage could be detected in such lysates to form plaques on RT 9 bacteria. This result, which taken alone suggests that RT 10 was a defective lysogenic strain, was difficult to reconcile with the demonstrated ready production of phage in the presence of the indicator strain RT 9.

Mechanism of Phage Occurrence in Mixed Cultures

The possibility that zygotic induction (11) of phage development occurred in mixed cultures of RT 9 and 10 was considered but was excluded by the following experiment. RT 9 was grown in broth in one limb and RT 10 in the other limb of a U-tube divided by a fritted glass plate (6), whose integrity to bacteria had been demonstrated in control experiments. After 48 hours' incubation about 10^8/ml of plaque-forming particles (on RT 9) were present in filtrates produced from each limb of the tube. This observation suggested that a filterable inducing agent occurred in association with one of these two bacterial cultures. Various combinations of indicator strains and potential agents of lysis were tested for lytic activity by cross-streaking the surface of agar. The results are summarized in Table I. RT 9 bacteria were lysed in the presence of RT 10 bacteria but not in the presence of RT 10 bacteria which had been killed by chloroform. Filtrates of RT 9 had no plaque-forming ability on either RT 9 or RT 10, but when RT 10 cells were preincubated with RT 9 filtrates, and then treated with chloroform, plaque-forming particles with the activity shown, respectively, on RT 9 or on RT 10 were found. From these results we conclude that RT 9 carried a filterable agent which did not form plaques on RT 10 but which induced the development of functional phage particles from RT 10 bacteria.

TABLE I

Cross-streak test of potential lysing agents on RT 9 or on RT 10 as indicators

Indicator strain	Agent tested	Visible lysis
RT 9	RT 10 cells	+
RT 9	RT 10 chloroform treated	−
RT 9	RT 10 + filtrate of RT 9*	+
RT 9	RT 9 + filtrate of RT 10*	−
RT 9	Filtrate of RT 9	−
RT 10	RT 10 + filtrate of RT 9*	+
RT 10	Filtrate of RT 9	−

*Mixture was incubated for 3 hours at room temperature, then treated with chloroform to kill bacteria.

The following experiment was designed to investigate changes in numbers of viable cells occurring when RT 9 and RT 10 grew together in mixed (liquid) culture; the results support the findings described above. RT 10 and a mutant

[3]Mitomycin C was obtained from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan.

of RT 9 resistant to 2000 μg/ml streptomycin were grown in broth together and separately. At intervals, samples were taken for the determination of numbers of viable cells and of plaque-forming particles. Viable cell counts were made with plain agar medium and also with agar containing streptomycin (1000 μg/ml). The number of streptomycin-sensitive RT 10 cells in the mixed culture was determined indirectly by subtracting the number of streptomycin-resistant colonies from the number of colonies which grew on plain agar; numbers of plaque-forming particles were determined in soft agar layer plates after treatment of samples with chloroform to kill bacteria. The changes in viable cell count in mixed and single cultures and also the number of plaque-forming particles (on RT 9) are illustrated in Fig. 1. A marked decrease in viable count of RT 10 in the mixed culture was observed during 3 hours' incubation, and death of RT 10 cells coincided with the appearance of functional phage particles forming plaques on RT 9. The surviving RT 10 cells increased in numbers thereafter and the cell count had reached the same level as the control culture at 24 hours. By contrast, the number of RT 9 cells in the mixed culture increased until the 7th hour and then remained almost constant.

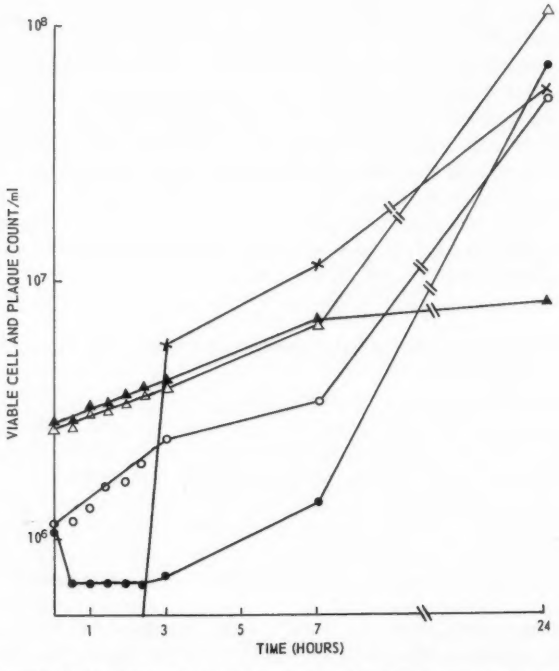

FIG. 1. Changes in numbers of viable bacteria and plaque-forming particles in mixed cultures of RT 9 and RT 10. ▲ RT 9 in mixed culture; △ RT 9 single culture; ● RT 10 in mixed culture; ○ RT 10 single culture; × plaque count made on RT 9.

Properties of the "Inducing" Agent

With the notable exception of lack of plaque-forming ability on any strain tested, the inducing agent liberated by RT 9 had properties similar to those of functional phage particles. For convenience in discussion, these particles will hereafter be referred to as "phage i".

Because phage i did not give rise to plaques it was necessary to use an indirect method of assay for quantitative studies. Serial dilutions of culture filtrates of RT 9 were mixed with equal volumes of cultures of RT 10 containing about 10^8 bacteria/ml. After a period of 3 hours at room temperature, to complete infection by phage i and liberation of induced functional phage, bacteria were killed by chloroform treatment and the contents of each serial dilution tube tested for the presence of functional phage, active on RT 9. From the highest dilution tube found to contain such phage the approximate titer of the original filtrate was calculated.

The chemical, physical, and morphological properties of phage i were similar to those of functional *Rhizobium* phages. Centrifugation at 27,000 *g* for 60 minutes reduced activity by 98%, as determined by the above test. Sensitivity to heat and to ultraviolet light were similar to those of the phage active on RT 9. Treatment with deoxyribonuclease and ribonuclease did not alter inducing activity. Attempts to obtain a purified preparation of phage i by ultrafiltration and differential centrifugation were unsuccessful. Nevertheless, phage-like particles (phage i) could be detected in ultraviolet-irradiated RT 9 culture when examined by electron microscopy as shown in Figs. 2 and 3. As a control functional phage active on RT 9 (phage 9) was also examined (Figs. 4, 5). Both phage i and phage 9 were spermlike with dense heads. However, phage 9 had heads which were definitely smaller than those of phage i.

It is interesting to note that the development of the hypothetical prophage i carried by RT 9 was induced by ultraviolet light. Cells of RT 9 were irradiated in saline with a 95% killing dose of ultraviolet light. Eighteen hours later it was noted that the irradiated culture had lysed and on examination it was found to have about 100 times greater inducing activity than that of unirradiated cultures. Similar results were obtained by growing logarithmic phase RT 9 cultures with mitomycin C (15) at concentrations of 1.0 to 0.1 μg/ml. About two hours after addition of mitomycin C to cultures, lysis commenced (as determined by turbidity measurements) and a concomitant increase in phage i titer was found. It can therefore be concluded that RT 9 is lysogenic carrying an inducible prophage (i) which is defective in the sense that no other host in which it can multiply by means of the lytic cycle was found.

Yield of Induced Functional Phage

In experiments (to be described later) designed to determine the rate of inactivation of phage i by antisera against phage 9, precise estimates of titers were made in retrospect from results obtained by a "single burst" (7) technique. Because this technique yielded relevant information about the inducing activities of phage i, some of the results will be described now, rather than later.

Filtrates of RT 9 containing approximately 10^7 particles/ml were mixed

1:1 with cultures of RT 10 containing about 10^8 bacteria/ml in the presence of 100 μg/ml chloramphenicol. After 30 minutes for adsorption the mixture was diluted to a predetermined level and fifty 1-ml samples of the final dilution were distributed to test tubes, so as to obtain less than one phage-yielding bacterium per tube. After 4 to 6 hours a drop of chloroform was added to each tube and later the presence of functional phage tested for by plating 0.3-ml samples in soft agar layer with RT 9 as indicator. Previous experiments modeled on the single-step growth curve technique (1) for the study of phage multiplication showed that, following infection of RT 10 by phage i, a latent period of 2 hours occurred before plaque-forming (phage 9) particles were detectable in the mixture. Concurrently with these other operations, viable counts were made on the adsorption mixture to measure the killing effects of the phage i particles. Distributions of numbers of phage 9 particles resulting from the lysis of one or a small number of i-infected RT 10 bacteria are given in Table II. These data are not exact because 0.3-ml samples were plated rather than the whole 1-ml volume from each growth tube. Nevertheless, they indicate that the yield of phage 9 from each infected bacterium was of similar magnitude to that found in other and more orthodox phage-yielding systems (1). This observation suggests that the action of phage i is inducing

TABLE II

Distribution of numbers of phage 9 particles* resulting from lysis of one or a small number of RT 10 bacteria infected with phage i

No. of plaque-forming particles per tube												Mean no.† phage-yielding bacteria/tube
0	1–10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	Over 90	Total	
22	3	2	2	6	6	2	1	4	0	1	49	0.80
19	2	5	7	9	1	2	1	2	1	1	50	0.95

*RT 10 bacteria infected with phage i at multiplicity of about 0.1 phage/bacterium and diluted to obtain less than 1 infected bacterium/ml. Samples (1 ml) were distributed to tubes for bursting to occur, chloroform-treated, and samples plated on RT 9 soft agar layer plates. Entries in table are number of tubes which yielded, at a single sampling of 0.3 ml, a number of plaques falling within the stated range.

†Calculated by means of the Poisson formula $P_0 = e^{-z}$ where P_0 is the proportion of tubes with no phage and z is the required average number of infected bacteria/tube.

and that the production of functional phage does not depend on rare recombinational events occurring during phage multiplication. The estimated mean burst size based on these data was about 100 phage 9 particles per infected bacterium; this result agrees with an estimate obtained in a single-step growth curve experiment. For a given phage i particle lysate, similar retrospective estimates of titer were obtained by means of the dilution end-point method, from "single burst" experiments, and from the drop in viable counts of bacteria exposed to phage i particles. This last observation suggests that, as might be expected, the killing and inducing activities of phage i were closely connected.

FIGS. 2 and 3. Electron photomicrographs of phage i.
FIGS. 4 and 5. Electron photomicrographs of phage 9.

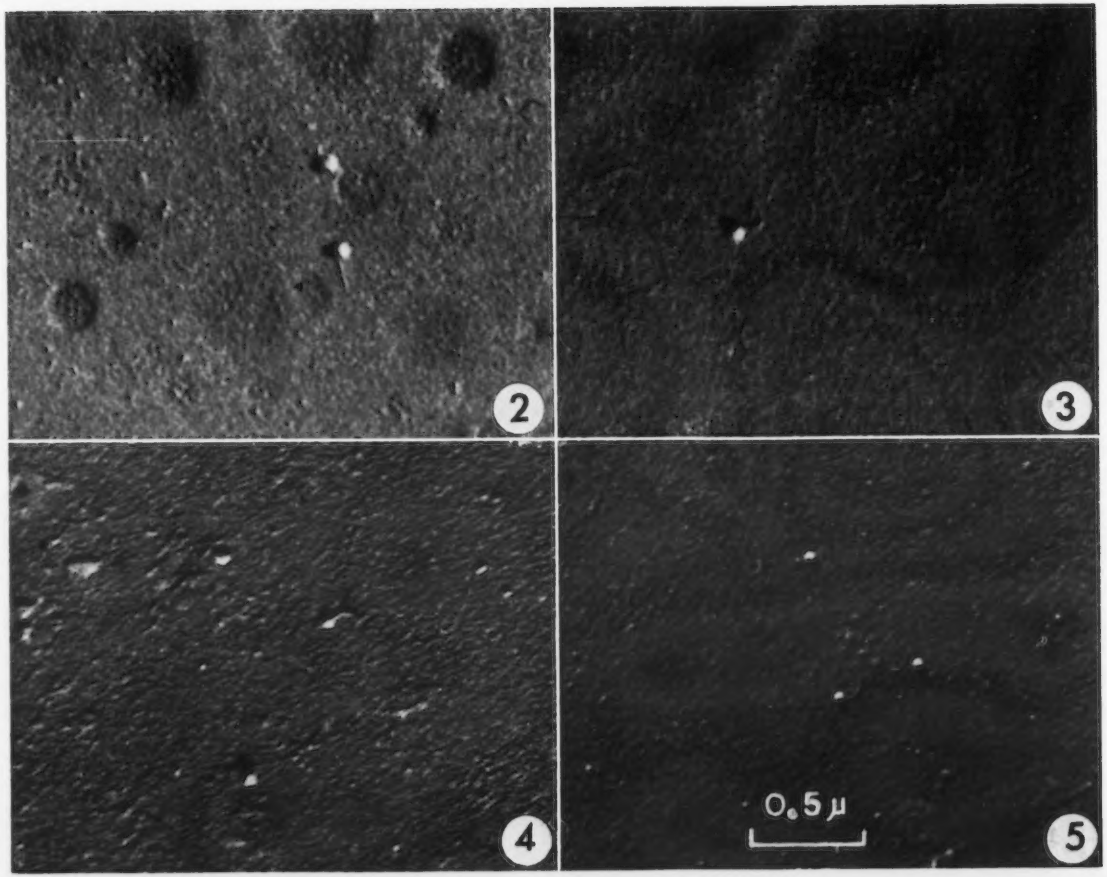

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PLATE II

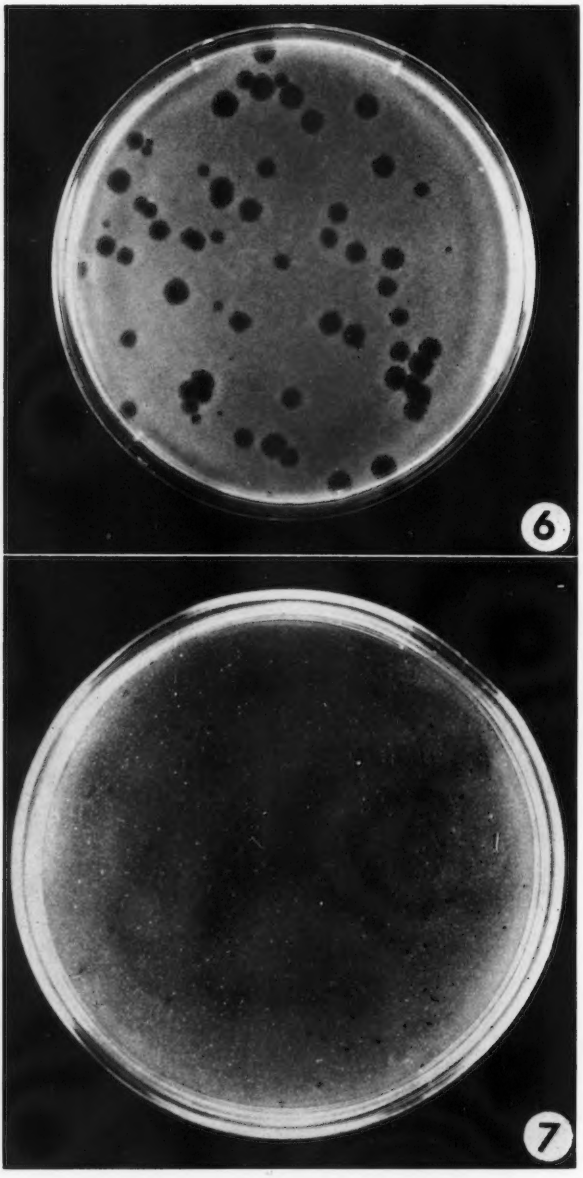

FIG. 6. Plaques produced on RT 9 by phage 9.
FIG. 7. Plaques produced on RT 10 by phage 10.

Host Range of Functional Phage Particles

When a culture of RT 10 containing about 10^8 cells/ml was mixed with an equal volume of RT 9 culture filtrate and incubated at room temperature the viable count of RT 10 dropped by about 30% within a period of 3 hours. Concurrently about 10^8 particles/ml producing plaques on RT 9 (phage 9) and about 10^2 particles/ml producing plaques on RT 10 (phage 10) were found in filtrates of this mixture. Undoubtedly, the rather low number of phage 9 particles found resulted from the readsorption of liberated phage by the large number of intact RT 10 bacteria still present and by bacterial debris present under these conditions. RT 10 bacteria are able to adsorb phage 9 although they are immune to infection by phage 9. As shown in Fig. 6, phage 9 forms distinct but turbid plaques on RT 9, whereas phage 10 (Fig. 7) forms smaller turbid plaques on RT 10. Phage 9 could be purified readily by picking

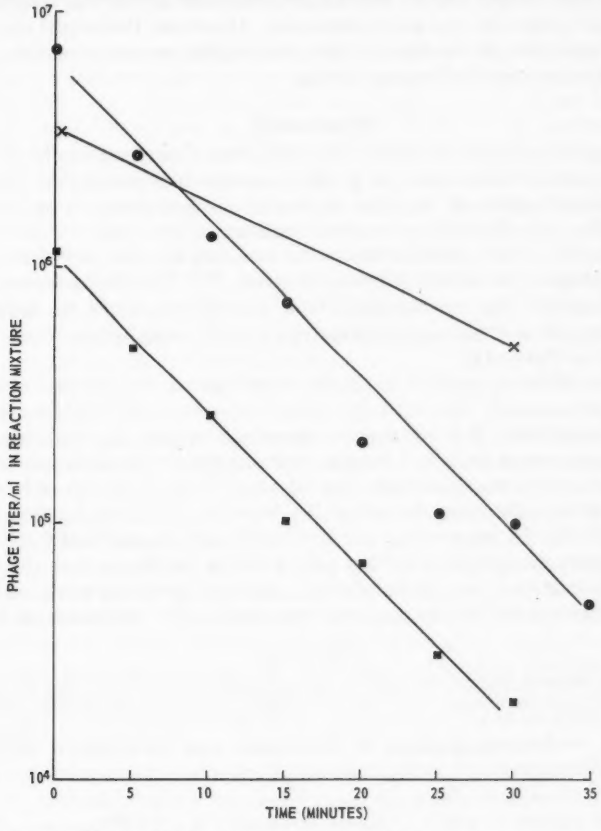

FIG. 8. Inactivation at 37° C of phages 9, 10, and i by antiserum prepared against phage 9. ● Phage 9; ■ phage 10; × phage i.

single plaques and was thereafter stable in that no phage 10 was detectable. However, cultures of RT 10 lysed by phage 10 always contained a small proportion (about 10^{-3}) of phage 9 particles, even after repeated reisolations.

To clarify the relationship between phages 9, 10, and i, a serological study was undertaken. Rabbit antiserum against a high titer lysate of phage 9 was prepared and the rates at which this serum inactivated the various phages determined as described by Adams (1). Mixtures of the respective phages and antiserum at 1/1000 dilution were incubated at 37° C and samples were taken at intervals for phage assay in order to determine the proportion of particles inactivated by the antiserum. As shown in Fig. 8, both phages 9 and 10 were inactivated by antiserum prepared against phage 9 and the slopes of the inactivation curves for these phages were indistinguishable. This result indicates that phages 9 and 10 are serologically related. By contrast, phage i assayed by the "single burst" technique described above was inactivated to a much lesser extent by the same antiserum. However, this slight inactivation of phage i may simply be due to the unavoidable presence of this phage in the RT 9 lysate used for immunization.

Discussion

The lysogenic *Rhizobium* strain (RT 10) first described by Marshall (14) was found, on examination, to possess unexpected properties. Ultraviolet irradiation resulted in cell lysis but no free phage was detected in such lysates or in filtrates of unirradiated control cultures. The presence of prophage elements in RT 10 is nevertheless demonstrated by the fact that upon infection by phage i, for which RT 9 is lysogenic, RT 10 cells burst and liberate phages 9 and 10. For convenience the inferred relationships between the bacterial strains and various demonstrated and hypothetical viral elements are set out in Table III.

RT 10 must be presumed to carry prophage of exceptional stability in that no spontaneously liberated free phage was ever detected in our experiments. Alternatively, RT 10 must be presumed to carry a prophage which is defective in the sense that it is unable, without external assistance, to embark on vegetative reproduction while carried by RT 10, although able to confer immunity to superinfection by phage 9. Defective lysogenic bacteria occur in nature (4, 8, 9). In most cases the carried phage is genetically defective in that no mature phage particles are produced on induction but the defective viral elements are maintained indefinitely as prophage in the so-called defective lysogenic bacteria (9). At the moment any explanation advanced for the origin

TABLE III

Relationships inferred between bacterial strains and both latent and mature bacteriophages

Strain	Carries prophage	U.V.-inducible	Liberates mature phages	Attacked by phages
RT 9	i	+	i	9
RT 10	9	−	9,* 10*	i, 10

*Liberated only after infection with phage i.

of phages 9 and 10 from RT 10 bacteria is speculative rather than conclusive. At least two different but not mutually exclusive concepts deserve consideration: (*a*) That phages 9 and 10 result from host-induced, presumably recombinational, events between infecting phage i and prophage elements carried by RT 10. These events cause changes in phage host-range and serological properties. (*b*) The second concept requires that the genetic material of phage i is active physiologically as an inducing agent when injected into RT 10 bacteria but is not active genetically. An analogy for such a situation could be sought in the phenomenon of abortive transduction (16, 22). Evidence has been obtained by these and other authors for the phage-mediated transfer of bacterial genetic determinants which are physiologically active but are not replicated at bacterial division. Either concept *a* or *b* (above) would require that phage i supply determinants for functions for which the hypothetical prophage 9 is presumed defective within the genetic background of bacterial strain RT 10.

The nature of the minority component (phage 10) liberated by RT 10 bacteria after infection with phage i is not clear. The results of the serological study suggest that phages 9 and 10 are closely related if not identical. At least two possibilities exist: phage 10 may be a host range mutant of phage 9 or it may originate in a recombination event between phage 9 and elements of phage i during multiplication in RT 10. Jacob and Wollman (10) found that an ultraviolet-irradiated virulent mutant of phage lambda induced prophage in *E. coli* and also gave rise to various recombinants. The fact that phage 9 did not seem to give rise to variants which form plaques on RT 10 when it multiplied in RT 9 remains unexplained. It is interesting to note that phage 10 when multiplying on RT 10 always liberated a small (10^{-3}) proportion of particles which behaved like phage 9. Phage 10 might therefore have slight (when compared with phage i) inducing power resulting from a changed host range determinant or from other, possibly recombinant, determinants.

Strain RT 9, previously reported (14), as an indicator strain, was unexpectedly found to be lysogenic for phage i. The evidence shows that this strain carries an inducible prophage and that with the exception of its lack of plaque-forming ability this phage had properties comparable to those of functional *Rhizobium* phages. The most interesting property of phage i is its inducing and concomitant lethal action on RT 10, which can legitimately be considered a defective lysogenic strain. While phage i could be called defective for the purpose of the present report, it is quite possible that indicator strains exist on which it could multiply normally by means of the lytic cycle.

The appearance of "new phage" in mixed cultures of *Shigella* and *E. coli*, both originally supposed free from phage, was reported in 1922 by Lisbonne and Carrère (12). Recently Sholtens (20) found phages in mixed cultures of *Salmonella* which were not detectable in either strain when grown alone. Coetzee and Sacks (3) made similar observations in *Proteus*. These observations are formally similar to ours, which suggests that similar mechanisms may be involved. The new phages in mixed cultures of enteric bacteria were usually detectable after a few days' incubation but the latent period for the

induction of RT 10 by phage i was about two hours. This difference may reflect differences in the frequency of occurrence of induction or of recombination; we were fortunate in that phage i was liberated spontaneously from RT 9 at a relatively high frequency.

The induction of development of the infectious phase of an otherwise latent virus has also been observed in insects. Smith and Rivers (21) observed the development of a cytoplasmic polyhedrosis (which had apparently been carried in latent form) on infection of insects with an unrelated virus.

The existence of a prophage, undetected by orthodox techniques, was reported by Rutberg and Hedén (19). They obtained functional phage from protoplasts of *E. coli* B by treatment with high pressures. Cohen (4) found dismune phage in lysates of *E. coli* B lysed by phage P2. This dismune phage, which was interpreted as a hybrid between P2 and a previously undetected prophage in *E. coli* B, was unable to lyse *E. coli* B but produced plaques on *E. coli* C lysogenic for P2. The above and other observations (9) together with our own on *Rhizobium* suggest that latent viral elements in nature may occur more frequently than hitherto realized.

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ÉTUDE DU VIRUS DE L'INFLUENZA

ANTIGÉNICITÉ DE SOUCHES GRIPPALES ADAPTÉES À DES CULTURES DE TISSUS[1, 2]

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Abstract

This paper deals with a study of the antigenic properties of influenza strains adapted to tissue cultures. When adapted to chick embryonic tissue, influenza strains lose most of their antigenicity; when adapted to monkey kidney cultures under the same conditions, influenza strains maintain their antigenic value to a high level.

Introduction

Un vaccin grippal développé sur culture de tissus peut avoir un grand intérêt soit pour la vaccination des personnes allergiques à l'oeuf, soit au moment de pandémies comme celle de 1957 où il est difficile de se procurer rapidement l'énorme quantité d'oeufs de qualité nécessaires à une production accélérée d'un vaccin contenant la nouvelle souche.

Cependant une expérience de Jensen (3) montre une baisse de l'antigénicité des souches grippales adaptées à des cultures de tissu. Cette observation est basée sur une étude où le virus grippal a été cultivé sur un seul tissu, la membrane chorio-allantoïque de l'oeuf embryonné de poule. Il était permis de penser que des souches grippales adaptées à d'autres tissus pouvaient présenter un comportement différent. Ce travail a donc été poursuivi pour vérifier cette hypothèse.

Matériaux et Méthodes

1. Souches virales utilisées

A. Souches adaptées à l'oeuf embryonné de poule

Différentes souches de virus ont été cultivées dans l'oeuf embryonné de poule. Le liquide allantoïque recueilli a servi d'antigène. Les souches utilisées sont les suivantes:

A PR8/34 E 198 M 593 E 169.

A2 Ri_2, Ri_3, Ri_4^+, Ri_5^-, Ri_5^+ (souches aimablement fournies par Mr. P. W. Choppin MD du Rockefeller Institute N.Y.).

A2 1751, souche asiatique isolée à l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Canada.

B. Souches adaptées à des cultures de tissu

Ces mêmes souches adaptées à l'oeuf ont été par la suite passées sur des cultures de rein de poulet. Dès le deuxième passage, certaines souches ont provoqué une dégénérescence des cellules et ont montré une réponse positive

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Contribution de l'École d'Hygiène et de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal, Montréal, Qué. Une partie de ce travail a été présentée au 28ième Congrès de l'ACFAS, octobre 1960.
[2]Cette recherche a été partiellement subventionnée par le Ministère de la Santé Publique de Québec (subvention fédérale-provinciale à la recherche sur la santé publique).

à l'épreuve d'hémadsorption (5). Ce sont donc ces dernières souches que nous avons choisies pour l'étude de leur antigénicité après avoir effectué plusieurs passages successifs sur ce tissu.

Les souches adaptées à l'oeuf ont été également cultivées sur rein de singe. En général dès le premier passage, une épreuve d'hémadsorption positive (1) et une cytopathogénicité intense ont été notées. Ces souches ont subi par la suite plusieurs passages consécutifs sur ces tissus. Ces souches virales adaptées à l'oeuf ou aux cultures de tissu ont été inactivées de deux façons:

(a) Formolage.—Le liquide allantoïque contenant le virus a été mis en contact avec du formol à la concentration finale de 1/5000 durant 72 heures à 10° C. Après ce laps de temps, le formol n'a pas été neutralisé et le titre infectant pour l'oeuf s'est montré négatif quoique le titre hémagglutinant soit resté stable. Pour les souches adaptées aux cultures de tissu, le traitement a été identique et l'épreuve de cytopathogénicité s'est également montrée négative.

(b) Beta propiolactone.—Les souches adaptées à des cultures de rein de singe ont aussi été traitées à la bêta propiolactone (Testagar & Co. Inc., Detroit, Michigan). La suspension de virus dans le milieu M150 a été gardée 96 heures à 10° C en présence de ce produit à la concentration finale de 1/10000. Après ce laps de temps, le virus a été complètement inactivé et nous n'avons observé aucune multiplication de virus sur les cultures de rein de singe.

C. Vaccin commercial

Un vaccin formolé, polyvalent préparé par L'Institut de Microbiologie et d'Hygiène de l'Université de Montréal a également été utilisé au cours de cette étude. Ce vaccin cultivé sur l'oeuf renferme les quatre souches suivantes dans les proportions indiquées:

A2 Australie/57	200 CCA
A PR8/34	100 CCA
A1 PR 301/54	100 CCA
B GL/54	100 CCA.

2. Titrage des anticorps

Les anticorps du sérum des souris immunisées ont été mesurés par la technique de l'inhibition de l'hémagglutination (7). Les sérums de 10 ou 12 souris de chaque groupe ont été mélangés et traités à la trypsine et au KIO_4 (6), pour éliminer les inhibiteurs non spécifiques de l'hémagglutination.

3. Immunisation des souris

Chaque groupe, formé de 30 souris, a été inoculé par voie intrapéritonéale (exp. 1 et 3) ou souscutanée (exp. 2) avec 1 ml des suspensions virales vivantes ou inactivées. Une deuxième inoculation identique a été répétée à 14 jours d'intervalle dans la troisième expérience.

Dix jours après la dernière administration de l'antigène, 10 souris de chaque groupe ont été saignées et le taux moyen des anticorps a été mesuré dans le mélange de ces 10 sérums. Les 20 souris qui restaient dans chaque groupe ont été inoculées, sous anesthésie, par voie nasale avec différentes doses mortelles d'une souche asiatique homologue adaptée à la souris.

Quatorze jours après cette dose d'épreuve, le pourcentage de survie des souris a été noté.

Résultats

Expérience 1

Dans une première expérience, l'antigénicité des souches A PR8/34 et A2 Ri_2 adaptées à l'oeuf embryonné de poule a été comparée à l'antigénicité des mêmes souches adaptées à une culture de rein de poulet. Pour comparer la valeur antigénique de ces souches, nous les avons diluées de telle sorte qu'elles contiennent le même nombre d'unités infectantes pour l'oeuf, soit: $10^{3.5}$ EID/50 par ml. Ces suspensions ont été inoculées vivantes.

Les résultats de cette expérience (tableau I) semblent confirmer les conclusions de Jensen (3). En effet, les deux souches utilisées protègent trois fois moins les souris après qu'elles ont été adaptées à une culture de rein de poulet.

TABLEAU I

Antigénicité de souches grippales adaptées à une culture de rein de poulet

Souches	Adaptation		EID/50 par ml $\log_{10}$*	Inhibition de l'hémagglutination (titre du sérum des souris immunisées†	Protection (% survie des souris à 100 LD/50 de la souche homologue)‡
	Hôte	Nombre de passages			
A PR8/34	Oeuf	166	8.7	80	66.0
A PR8/34	Rein de poulet	11	7.0	20	25.0
A2 Ri2/57	Oeuf	7	8.0	80	83.4
A2 Ri2/57	Rein de poulet	8	3.5	0	27.3
Témoins§				0	0.0

*La suspension inoculée par voie intrapéritonéale a été diluée pour contenir $10^{3.5}$ EID/50 par ml.
†Titre moyen du sérum de 10 souris sacrifiées 10 jours après l'inoculation de l'antigène par voie intrapéritonéale.
‡Pourcentage de survie de 20 souris immunisées 14 jours après l'inoculation par voie nasale, sous anesthésie, de 100 LD/50 d'une souche homologue.
§Souris non immunisées.

Expérience 2

Dans une deuxième expérience, cinq souches asiatiques adaptées à des cultures de rein de singe ont été étudiées. Ces suspensions virales ont été subdivisées en trois parties traitées comme suit:

(*a*) Virus non traité (vivant),
(*b*) Virus traité au formol,
(*c*) Virus traité à la bêta propiolactone.

Les résultats de cette expérience (tableau II) montrent que les souches adaptées aux cellules de rein de singe donnent une protection du même ordre que les souches adaptées à l'oeuf (tableau I). Ici le virus vivant donne la même protection que le virus traité au formol ou à la bêta propiolactone, ce qui est différent des résultats que nous avons trouvés dans d'autres travaux (2). Nous pouvons encore noter que le titre infectant pour l'oeuf des souches adaptées à des cultures de rein de singe ne semble pas avoir de relations avec leur valeur antigénique. Le nombre de passages sur culture de tissu ne semble pas non plus affecter le pouvoir antigénique des souches, au moins dans les limites de l'expérience.

Expérience 3

Dans une troisième expérience, nous avons comparé la valeur antigénique

TABLEAU II

Antigénicité de souches grippales adaptées à une culture de rein de singe

Souches	Adaptation au rein de singe: Nombre de passages	Adaptation au rein de singe: EID/50 par ml log_{10}*	Traitement du virus	Inhibition de l'hémagglutination (titre du sérum des souris immunisées)†	Protection (% survie des souris à 100 LD/50 de la souche homologue)‡
Ri3	15	6.0	Aucun	160	57
Ri3	15	6.0	Formol	160	44
Ri3	15	6.0	β propiolactone	320	69
$Ri4^+$	7	3.6	Aucun	320	69
$Ri4^+$	7	3.6	Formol	160	69
$Ri4^+$	7	3.6	β propiolactone	40	69
$Ri5^-$	16	3.5	Aucun	640	69
$Ri5^-$	16	3.5	Formol	160	82
$Ri5^-$	16	3.5	β propiolactone	40	57
$Ri5^+$	10	5.3	Aucun	80	69
$Ri5^+$	10	5.3	Formol	80	69
$Ri5^+$	10	5.3	β propiolactone	40	44
A2 1751	9	6.0	Aucun	40	82
A2 1751	9	6.0	Formol	20	44
A2 1751	9	6.0	β propiolactone	160	69
Témoins§				0	0

*Suspensions inoculées sans dilution; un ml par voie souscutanée.
†Titre moyen du sérum de 10 souris sacrifiées 10 jours après l'inoculation de l'antigène par voie souscutanée.
‡Pourcentage de survie de 20 souris immunisées 14 jours après l'inoculation par voie nasale, sous anesthésie, de 100 LD/50 d'une souche homologue.
§Souris non immunisées.

TABLEAU III

Antigénicité d'un vaccin polyvalent produit sur l'oeuf, comparée à celle d'une souche asiatique adaptée sur culture de rein de singe

Antigène	Adaptation: Hôte	Adaptation: Titre initial*: CCA/ml	Adaptation: Titre initial*: EID/50/ml log_{10}	Traitement du virus	Inhibition de l'hémagglutination (titre du sérum des souris immunisées)†	Protection (% survie des souris à 100 LD/50 de la souche homol.)‡: 10	100	1000
Vaccin§ I.M.H.	Oeuf	200	8.0	Formol	550	100	100	95
A2 1751	Rein de singe‖	8	3.5	Formol	150	90	90	5
A2 1751	Rein de singe‖	8	3.5	Bêta propiolactone	280	75	50	25
Témoins¶					0	0	0	0

*Avant le traitement du virus. Suspensions inoculées non diluées par voie intrapéritonéale.
†Titre moyen du sérum de 10 souris sacrifiées 10 jours après l'inoculation de l'antigène par voie intrapéritonéale.
‡Pourcentage de survie de 20 souris immunisées 14 jours après l'inoculation par voie nasale, sous anesthésie, de 10, 100, 1000 doses LD/50/ml d'une souche homologue.
§Vaccin polyvalent produit par l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal et composé de quatre souches:

A2 Australie/57	200 CCA/ml
A PR8/34	100 CCA/ml
A1 PR 301/54	100 CCA/ml
B GL /54	100 CCA/ml.

‖Treizième passage.
¶Souris non immunisées.

d'un vaccin polyvalent formolé adapté à l'oeuf et d'une souche asiatique adaptée à une culture de rein de singe traitée au formol ou à la bêta propiolactone.

Cette fois le vaccin polyvalent adapté à l'oeuf semble plus efficace que la souche adaptée à des cellules de rein de singe (tableau III). Toutefois, si on considère la protection obtenue contre 100 doses mortelles de virus, cette différence de protection entre ce vaccin et la souche adaptée au rein de singe se situe dans les limites du taux de protection trouvé pour les souches adaptées à l'oeuf (tableau I), protection qui varie de 60% à 100%.

Il est reconnu que dans un vaccin antiinfluenza polyvalent, l'antigénicité de chacune des souches est augmentée si on augmente le nombre des souches du vaccin (4). Ce facteur peut expliquer que le pourcentage de survie des souris soit plus élevé avec le vaccin polyvalent qu'avec les souches individuelles.

Le vaccin polyvalent est un vaccin concentré et nous n'avons pu lui comparer quantitativement le virus adapté à des cultures de rein de singe. En effet les souches perdent graduellement leur pouvoir agglutinant et leur infectivité pour l'oeuf quand on les adapte à des cultures de tissu, les unités ne sont plus les mêmes et par conséquent nous n'avons pu comparer la quantité de particules virales inoculées dans les deux cas.

Discussion et Conclusions

Les résultats obtenus au cours de ces expériences ne permettent pas de tirer une règle générale sur le comportement antigénique des souches du virus grippal adaptées à des cultures de tissu. Alors que des souches adaptées à des cultures de rein de poulet ont perdu les deux-tiers de leur pouvoir antigénique, d'autres souches adaptées à des cultures de rein de singe ont conservé ce pouvoir presqu'au niveau initial. Il est donc évident que le degré d'antigénicité conservé dépend du choix des tissus employés et peut-être aussi des souches utilisées.

Il nous a été impossible de comparer quantitativement une dose de virus adapté à l'oeuf avec une dose de virus adapté à des cultures de tissu; dans le premier cas il s'agit d'unités CCA ou EID/50 et dans le deuxième cas d'unités cytopathogéniques ou hémadsorbantes (1). La seule unité commune serait précisement basée sur la qualité antigénique des différentes souches.

Les suspensions virales traitées à la bêta propiolactone ont donné des titres du même ordre que les suspensions virales traitées au formol. L'utilisation de ce produit chimique dans l'inactivation du virus est intéressante puisque l'antigénicité du virus est conservée et qu'il n'y a pas lieu de dialyser la suspension après cette opération pour effectuer les épreuves d'inocuité sur cultures de tissu, l'hydrolyse de la bêta propiolactone ne donnant pas de résidus toxiques.

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IMMUNOCHEMICAL STUDY OF THE ANTIGENS OF TRICHINELLA SPIRALIS

I. IDENTIFICATION AND ENUMERATION OF ANTIGENS[1]

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Abstract

Antigenic analyses of extracts of *Trichinella spiralis* larvae were done using immunoelectrophoresis as the principal analytical method. Eleven electrophoretically distinct antigens were identified in buffered saline extracts. Different numbers of antigens (but not more than 11) were found in other preparations, but these gave reactions of complete immunological identity with antigens in the saline extract.

Trichinella spiralis enters the host by way of the digestive tract as larvae encysted in meat. The larvae are released from their cysts by the gastric fluid and pass into the intestinal tract where they penetrate into the mucosa. There they undergo rapid growth, and mating takes place as early as 48 hours after ingestion. The larvae are deposited in the tissue of the intestine about one week later and enter the blood and the lymphatics. The larvae are carried to the skeletal muscles of the host, develop further, roll themselves into a spiral, and encyst.

Although the antibody response of the host to the parasite has been used for the serological diagnosis of trichinosis (4, 7, 9, 15, 20, 21), false positive and cross reactions have been reported (1, 2, 19, 24). In order to minimize these non-specific reactions, attempts have been made to isolate a specific antigen from *T. spiralis* larvae (13, 14, 20, 24). These preparations have given more reliable serological tests.

The number of antigens in these purified preparations has received some study. Wodehouse (24) has reported that saline extracts of trichinella larvae contain 10 antigens, and Kagan and Bargai (12) found three antigens in an acid-soluble extract of infective larvae. These authors used the double diffusion method of precipitin analysis in agar.

An assay of the numbers of antigens in *T. spiralis* larvae was necessary as a prelude to studies on the nature of these antigens and on the specificity of the serological reaction. The assay was done in this present study using, for antigenic analysis, the immunoelectrophoretic method described by Grabar and Williams (8). Several different antigenic preparations have been examined in this way and an immunological comparison has been made of the antigens in these preparations. An estimate of the complexity of the antigenic make-up of the larvae has also been made using in vitro immune precipitation.

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Experimental Methods

Isolation of Larvae from Infected Muscle

Minced infected rabbit muscle is digested in an artificial gastric fluid composed of 0.5% HCl (v/v) and 0.3% pepsin (w/v) in tap water at 37° C with constant agitation. After 4 hours the mixture is filtered through gauze and the larvae, freed from their cysts, are allowed to sediment and are harvested with a Pasteur pipette. After three washings with phosphate-buffered saline (pH 7.2), the larvae are counted and used for the infection of other animals or washed three more times with glass-distilled water, lyophilized, and used for antigen extraction.

Infection of Rabbits

Freshly digested, washed, and counted larvae in a buffered saline suspension are forcibly fed to rabbits. One month following infection the animal is exsanguinated and its muscle digested. The strain of *Trichinella spiralis* has been maintained at the Institute of Parasitology for a number of years in rats and has been passaged through rabbits.

Extraction of Antigens from T. spiralis Larvae

Lyophilized larvae, separated from infected muscle by peptic digestion, were weighed and suspended in phosphate-buffered saline (pH 7.2) in the proportion of 1 g of larvae to 100 ml of fluid. This suspension was homogenized for 15 minutes with a Servall high speed "Omni-Mixer" in a stainless steel chamber containing a small quantity of Pyrex glass particles. During homogenization the stainless steel container was immersed in an ice bath to reduce possible protein denaturation. The resulting suspension, which contained only small fragments of larvae, was centrifuged at room temperature for 20 minutes at 7000 r.p.m. in a Servall "SS-1" centrifuge with a 9-in. rotor. Merthiolate in a concentration of 1:10,000 was added to the supernatant and this was stored at 5° C. This extract was labelled 1:100 Trichinella Saline Extract.

Other antigenic extracts of lyophilized larvae were prepared according to the methods proposed by Melcher (14) and Suessenguth and Kline (20).

The metabolic antigen preparation of *T. spiralis* larvae was a dialyzed* and twice-concentrated medium in which larvae had been maintained alive for 5 days. The medium contains 29 amino acids, various vitamins and growth factors, dextrose, and glycogen in Hank's inorganic salt solution (16). The pH of the medium is 7.0.

Immunoelectrophoretic Analysis

Immunoelectrophoresis was performed according to the method proposed by Grabar and Williams (8) or to the micro modification of this procedure as described by Scheidegger (18).

One per cent Difco Noble Agar, containing Veronal buffer (pH 8.6, $\mu = 0.0375$) (3), was poured over glass plates or microscope slides to give a convenient depth of agar on the glass supports (2 mm deep over the glass plates, 1–1.5 mm over the microscope slides). After the agar had gelled, wells were

*The medium was dialyzed for 24 hours against running tap water and for 4 days against several charges of $M/15$ NaCl solution.

cut into it at the center of the slide and filled with a 1:2 mixture of the antigen and 2% Difco Noble Agar in distilled water. The slides were then transferred to an electrophoresis apparatus of the type described by Grabar and Williams and a potential was applied across them. The glass plates (5 × 7 in.) were run for 5 hours at 500 volts, 40 ma, and the glass slides (1 × 3 in.) were run for 2 hours at 80 volts, 10 ma. After the electrophoretic segregation had been completed, a rectangular trough was cut into the agar parallel to the direction of electrophoretic migration and the trough filled with an appropriate antiserum. When the arcs of specific immune precipitation had fully developed, the plates were washed over a period of several days with several charges of *M*/15 NaCl solution to elute excess serum from the gel. When protein could no longer be detected in the wash fluid, the agar gel was dried to a film and the arcs of precipitation were stained with Amidoschwarz 10B (22).

In vitro Immune Precipitation Analysis

Immune precipitations were done according to the method described by Cohn (6). In this reaction, pooled rabbit serum was mixed with several different amounts of antigen. The volume of each reaction mixture was adjusted to 1.5 ml with pH 7.2 phosphate-buffered saline and the reactions incubated at 37° C for 1 hour and for 18 hours at 5° C. The immune precipitates obtained after this incubation period were sedimented by centrifugation at 9000 r.p.m. for 20 minutes at 4° C. The supernatant of each antigen–antibody mixture was analyzed for the presence of excess antigen or antibody by overlaying, in a small bore glass tube, the supernatant with the homologous antigen (for the detection of excess antibody) or the homologous serum with the supernatant (for the detection of excess antigen). An excess was indicated by a "ring" of specific immune precipitation at the interface of the two fluids.

Experimental Results and Discussion

Although there have been many reports concerning the preparation of antigenic extracts from *Trichinella spiralis* larvae, only a few investigators (12, 24) have attempted to analyze the numbers of antigens contained in such preparations.

In order to determine the antigenic complexity of these larvae, specific in vitro immune precipitations were done with a 1:100 trichinella saline extract and a pooled serum from experimentally infected rabbits (6). The supernatants of these precipitations were examined in the "ring" test for the presence of excess antigen or antibody.

The results of this determination are illustrated in Table I.

In the classical supernatant analysis after in vitro immune precipitations (6), zones of antigen or antibody excess are obtained, along with an equivalence zone where neither of these reagents can be detected. In systems containing only one antigen and one antibody, the equivalence zone can encompass several reaction mixtures.

An equivalence zone was not obtained in our precipitin reaction even though antigen and antibody were combined in such different proportions that an excess of each one of the reagents was obtained at the extremes of the

reaction series. The extensive overlapping of the antigen and antibody excess zones in the reaction of trichinella saline extract with immune rabbit serum suggested that the antigen preparation was immunologically complex (11).

TABLE I

Supernatant analysis of in vitro immune precipitation of a buffered saline extract of trichinella larvae and pooled immune rabbit serum*

ml TSE† per 0.5 ml serum	0.02	0.03	0.04	0.05	0.10	0.20	0.30	0.40	0.50	0.60	0.70	0.80	0.90	1.00
Antigen excess	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Antibody excess	+	+	+	+	+	+	+	+	+	+	+	+	−	−

*The antiserum was a pool of equal amounts of sera from 20 experimentally infected rabbits.
†Trichinella-buffered saline extract. See text for details of preparation.

Immunological reactions in vitro are very useful in the quantitative analysis of the antigen–antibody reaction. They cannot, except in relative and general terms, indicate the numbers of antigens and antibodies taking part in an immunological reaction. Since it was of interest to know the numbers of antigens in trichinella larval extracts, the complement of antigens in trichinella saline extract was determined using the method of immunochemical analysis described by Grabar and Williams (8).

Trichinella buffered saline extract was applied at four places at the center of an agar-covered glass plate 5 × 7 in. in size. The antigens in the preparation were separated by electrophoresis using a current strength of 40 ma at 500 volts for 5 hours in pH 8.6 Veronal-buffered 1% agar. After completion of the electrophoretic separation, the antigens present in the extract were revealed using, on the same plate, individual sera from several different trichinella-infected rabbits. The use of more than one antiserum permits the identification of antigens which may not be antigenic in one individual animal but may be so in another.

The result of one such determination is illustrated diagrammatically in Fig. 1. The sera of eight different trichinella-infected rabbits were used. A composite of the immune precipitation arcs obtained in this determination is also presented in Fig. 1. Each antigen in the saline extract detected by this method was labelled with a letter to facilitate its identification.

These results indicate that there are at least 11 electrophoretically distinct saline-soluble antigens in trichinella larvae. The electrophoretic mobilities of the constituents of this antigen preparation were related to the mobilities of the protein constituents of normal human serum. Using this convention there are four trichinella antigens with mobilities similar to gamma globulins (antigens A, B, C, and D), one that moves as a $beta_2$ globulin (antigen E), one that moves as a $beta_1$ globulin (antigen F), two that move as $alpha_2$ globulins (antigens G and H), two that move as $alpha_1$ globulins (antigens J and I), and one antigen that moves as serum albumin (antigen K).

Employing double diffusion precipitin analysis in agar, Wodehouse (24) found 10 different antigens, which he labelled from *a* to *j*, in a pH 6.8 extract of trichina larvae. Although Wodehouse says that he was ". . . uncertain

whether they should be regarded as ten different antigens. . . ", it would appear from these present results that they may correspond to the ones identified here and, therefore, are antigenically distinct one from another. It is difficult to relate the antigens revealed after double diffusion with those identified after immunoelectrophoresis. It would appear, however, that Wodehouse's band *i* is analogous to our antigen D since in both cases it is the "major" antigenic component of the extract.

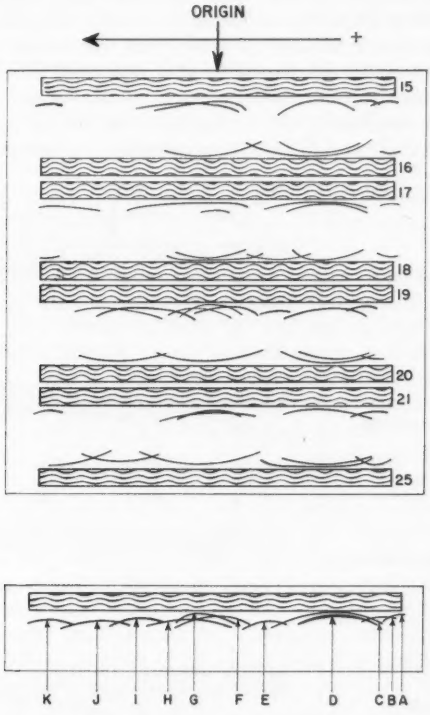

FIG. 1. Antigens in a pH 7.2 saline extract of trichinella larvae as revealed by eight different immune rabbit sera and composite diagram of the antigens in pH 7.2 saline extracts of trichinella larvae. Immunoelectrophoresis was done for 5 hours at 500 volts, 40 ma, in pH 8.6 Veronal-buffered 1% agar. Numbers identify the rabbit sera and letters identify the antigens in the extract.

In the years since an interest in the serology of trichinosis has been prominent, a number of antigenic preparations have been proposed as diagnostic aids for the disease. Of these many preparations, the fractions isolated by Melcher (14) have been among those most commonly used. Suessenguth and Kline (20) prepared an alkaline extract from *T. spiralis* larvae which they used for a slide agglutination test. In recent years some attention has been given to the metabolic products of these larvae (10, 17) as being of importance

in the immunity of the infected host. It was of interest, therefore, to determine what antigens are extracted by these methods and also to determine the nature of the similarity of antigens so extracted.

Immunoelectrophoresis of these antigen preparations was done using a modification proposed by Scheidegger (18). In this procedure microscope slides substitute for the glass plates as a support for the agar gel permitting smaller quantities of reagents to be used effectively and requiring less time for electrophoresis. The antiserum used to "develop" these immunoelectrophoreses was a pool of 20 sera from experimentally infected rabbits. Electrophoretic separation was achieved by subjecting the antigen preparations for 2 hours to a current of 10 ma per slide at a potential difference of 80 volts.

The results of these immunoelectrophoretic analyses are illustrated in Fig. 2. As illustrated in the figure, the antigens of the different preparations examined have electrophoretic mobilities similar to constituents identified

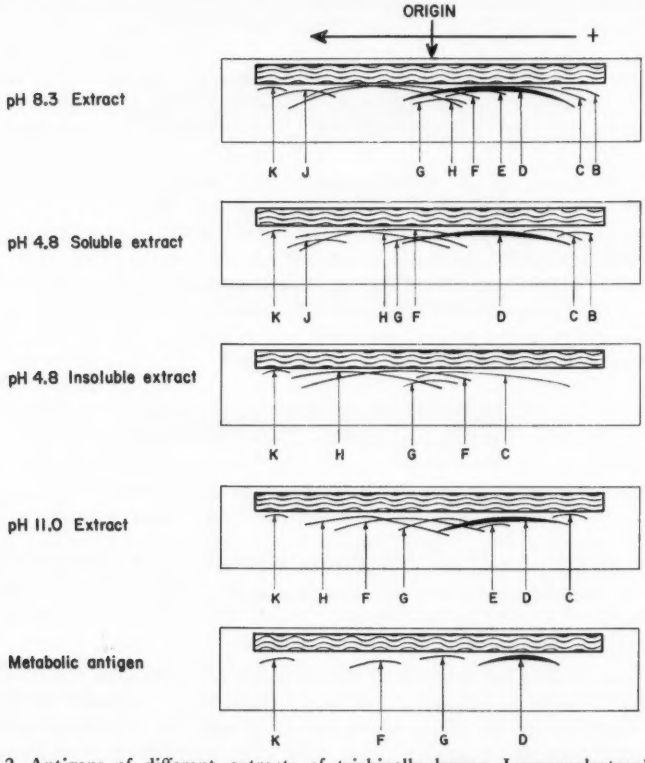

FIG. 2. Antigens of different extracts of trichinella larvae. Immunoelectrophoreses were done for 2 hours at 80 volts, 10 ma per slide, in pH 8.6 Veronal-buffered 1% agar, $\mu = 0.0375$. Immunoelectrophoreses were "developed" with a pool of equal amounts of sera from 20 experimentally infected rabbits.

in the buffered saline extract. For this reason these antigens have been labelled as those of the saline extract.

All the extracts examined in this study are crude preparations and, although there is a difference in the numbers of antigenic components, each extract is still relatively complex. In Melcher's purification procedure, the pH 8.3 extract is the first fraction obtained from petroleum ether extracted larvae. From this are derived the pH 4.8 soluble and the pH 4.8 insoluble extracts. Of the eight precipitating antigens in the pH 8.3 fraction, one (antigen E) is adversely affected by the subsequent acid treatment since it appears neither in the pH 4.8 soluble nor in the pH 4.8 insoluble extracts. Of the remaining seven antigens, three (antigens B, D, and J) are not precipitated at the low pH.

Extraction of *T. spiralis* larvae at pH 11.0 with sodium carbonate according to the method proposed by Suessenguth and Kline (20) yields six antigens, the electrophoretic mobilities of which are similar to antigens in other larval extracts. Although Melcher's initial extract and that of Suessenguth and Kline are alkaline preparations, the former isolates more antigenic constituents of the larvae.

Larvae which were maintained alive in a completely synthetic medium produced four antigens with recognizable electrophoretic mobilities.

Results of the immunoelectrophoretic analysis of four different antigenic preparations from *T. spiralis* larvae furnish some evidence of the stability of these antigens in acid or alkali. All these antigens are soluble in relatively mild (pH 8.3) alkaline conditions, but, when the pH is raised to 11.0, antigens B, C, and J become insoluble or are denatured. At pH 4.8 antigens B, D, and J are completely soluble whereas antigens C, F, G, H, and K are only partially soluble since they appear in both the precipitate and the supernatant. Antigen E is labile to acid treatment since it is found neither in the precipitate nor in the supernatant when the pH is adjusted to 4.8. It seems improbable that antigens C, F, G, H, and K could be easily separated from the antigenic mixture by simple acidic or basic or basic precipitation since they seem to be insensitive to such fractionation.

The electrophoretic similarity of the antigens extracted from *T. spiralis* by one method to those extracted by another method indicates that they are physicochemically related. It was of interest to determine whether these physicochemical similarities were a reflection of immunological identity.

Clausen and Heremans (5) have recently proposed an elegant method for detecting immunological relationships in immunoelectrophoresis. After completing electrophoresis of two antigen preparations in agar, the immunological identity of which is to be determined, the antiserum trough between them is made discontinuous. Immunological identity is determined as in the Ouchterlony agar diffusion precipitin analysis. If two individual arcs of precipitation join to form one continuous arc, the two antigens producing the arcs are immunologically identical; if, however, the arcs of precipitation do not join but cross one another, the antigens forming the arcs are immunologically different.

The application of this method to the analysis of extracts of trichinella

larvae is illustrated in Fig. 3. In order to have a base for comparison, immunological relationships were all made to the pH 7.2 buffered saline extract.

The results of this experiment indicate the identity of the antigens extracted by the different methods investigated. In no instance has there been any indication that acid or alkali extraction or the isolation of metabolic products yields antigens different from those found in the buffered saline extract. Reactions of complete identity are given by antigens in the extracts and the metabolic product with antigens of the saline extract.

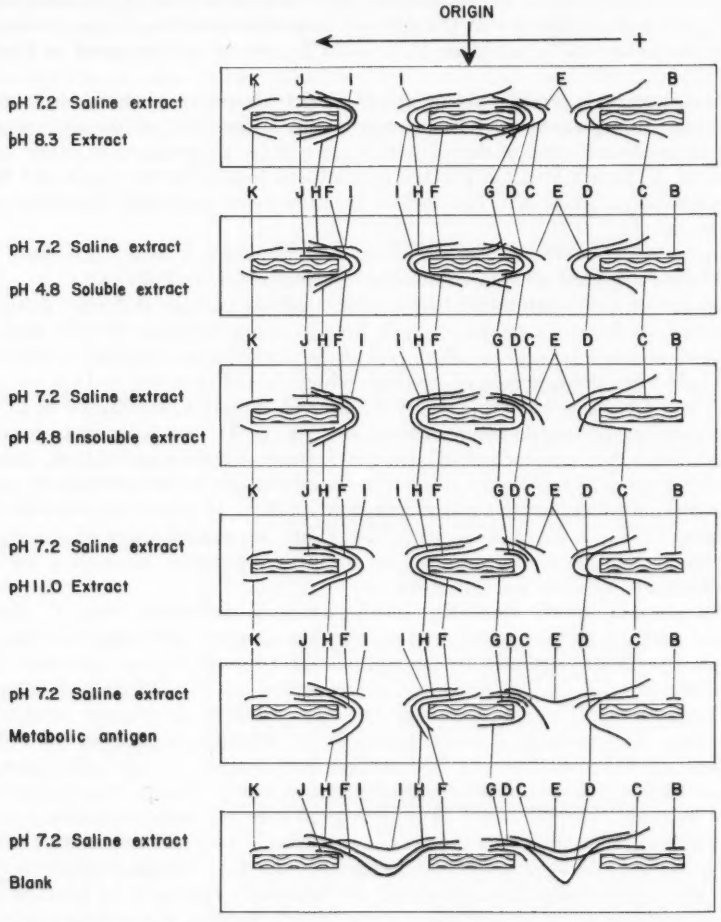

FIG. 3. Immunological identity of the antigens in different extracts of trichinella larvae. Immunoelectrophoreses were done for 2 hours at 80 volts, 10 ma per slide, in pH 8.6 Veronal-buffered 1% agar, $\mu = 0.0375$. Immunoelectrophoreses were "developed" with a pool of equal amounts of sera from 20 experimentally infected rabbits.

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RELATION OF TEMPERATURE AND SODIUM CHLORIDE CONCENTRATION TO GROWTH AND MORPHOLOGY OF SOME HALOPHILIC BACTERIA[1]

N. E. Gibbons and John I. Payne[2]

Abstract

The red halophiles, *Halobacterium salinarium*, *H. cutirubrum*, *H. halobium*, and *Sarcina litoralis*, grew most rapidly at salt concentrations of 20–25% and temperatures of 40–45° C. Maximum turbidity was obtained at similar salt concentrations but at 35–40° C. An unidentified colorless rod grew most rapidly at salt concentrations of 17.5–20% and temperatures of 40–50° C, but produced maximum yield at 30° C. The rod forms changed from long slender rods through irregular shapes to spheres as the salt concentration was decreased. At temperatures above the optimum, cells were very irregular, but otherwise temperature at any one salt concentration had little or no effect on the morphology.

Introduction

Different optimal temperatures have been reported for the growth of the extreme halophiles, *Halobacterium salinarium*, *H. cutirubrum*, *H. halobium*, and *Sarcina litoralis*. Harrison and Kennedy (9) reported that *Pseudomonas salinaria* grew optimally at 42° C, as stated in the sixth edition of Bergey's Manual. However, in the seventh edition of Bergey's Manual (4) the optimum temperature for the four organisms mentioned above is given as 37° C. Since cells of several species of these organisms were being grown routinely in these laboratories at 30°, it was decided to determine the effects of higher temperatures and of salt concentration on their growth and morphology, under what were considered better nutritional and cultural conditions than had been used previously. The results are presented in this report.

Materials and Methods

Four species of red-pigmented halophiles were used; *Halobacterium salinarium*, *H. cutirubrum*, *H. halobium*, and *Sarcina litoralis*, and an unidentified rod, designated as A2*c*, isolated from salted fish and producing an almost transparent, watery colony on salt agar. Cells were grown in a liquid medium containing casamino acids (Difco) 0.5%, yeast extract (Difco) 0.5%, trisodium citrate 0.3%, potassium chloride 0.2%, magnesium sulphate heptahydrate 2.0%, and sodium chloride as required (15.0–30.0%). The ingredients were dissolved in 80 ml of distilled water, the pH adjusted to 7.5–7.8 with *N* NaOH, and the medium autoclaved for 5 minutes at 120° C. It was then filtered to remove the precipitate, the pH adjusted to 7.4 with *N* HCl, and the volume made up to 100 ml.

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The organisms are very aerobic and to provide aeration without evaporation and subsequent change in salt concentrations the following method was devised. About 40 cm of 18-mm glass tubing was sealed onto 18-mm test tubes matched for use in the Coleman Jr. spectrophotometer. The broths containing 15 to 30% salt (NaCl) in 2.5% increments were dispensed (10 ml) into these elongated tubes and the tubes fitted with 5-mm glass tubing extending to the bottom and held in position at the top with plastic plugs (diSPo plug) split lengthwise. The liquid level was marked and the tubes sterilized. The tubes were inoculated by long Pasteur pipettes with a drop of a 4- to 5-day-old culture grown on a shaker in broth containing 25% salt. They were then incubated in racks in water baths maintained at temperatures of 30 to 55° C in 5° increments, and growth was followed by the change in optical density at 660 mμ. Air was passed through two gas-washing bottles containing 25% salt solution, connected in series to a manifold, both bottles and manifold being immersed in the water bath. The manifold allowed connections to the individual tubes. By humidifying the air at the temperature of incubation and with the long tubes acting as air condensers, there was no change in volume in any of the tubes during the experiments.

Results

Typical growth curves are presented in Figs. 1, 2, and 3. None of the red rods grew at 55° C and *S. litoralis* did not grow at 50° C. Based on these results, the following seem to be the optimal conditions of salt concentration and temperature of incubation for the organisms studied:

	For maximal rate of growth		For maximum turbidity	
	Temp. (°C)	Salt concn. (%)	Temp. (°C)	Salt concn. (%)
H. salinarium	45	25–30	35	25–30
H. cutirubrum	40	20–25	40	20–25
H. halobium	40	20–25	30–35	20–25
S. litoralis	40	20–30	40	20–30
A2*c*	40–50	17.5–20	30	17.5–20

It is quite possible that a greater yield of cells might have been obtained at lower temperatures had incubation continued.

H. cutirubrum and *H. halobium* were similar in their salt requirements and response to temperature. Growth was best at the optimum salt concentrations (20–25%) at all temperatures, except 50° C where no growth was obtained in 20% salt. In 15% salt the organism grew at temperatures of 30 and 35° C, but very little at higher temperatures; in 17.5% salt growth was reasonably good at temperatures up to the optimum.

Variable results were sometimes obtained, particularly at low salt concentrations. For example, in the experiment reported (Fig. 2) *H. salinarium* did not grow in 15% salt at any temperature and appreciable growth was obtained in 17.5 and 20% salt only at 35°. In other tests some growth has been obtained in 15% salt at 30 to 40°; growth at 17.5% was good at 40° but only slightly at 45°.

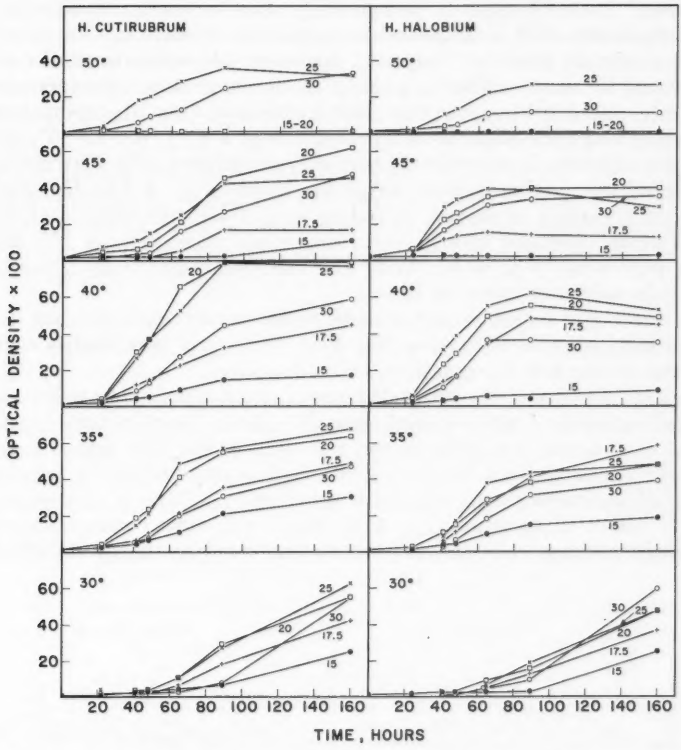


FIG. 1. Growth curves of *H. cutirubrum* and *H. halobium* at various temperatures and salt concentrations.

Growth of *S. litoralis* was characterized by long lag periods at all temperatures and the almost complete reversal of the optimal salt concentrations as the temperature increased from 30 to 45° C (Fig. 2). The optimum salt concentration therefore seems to depend on the temperature, but around 35 and 40° it is about 20%.

The rod A2*c* grew well at 30° after a fairly long lag and was the only organism tested which showed any growth at 55° (Fig. 3). Its optimal salt concentration (17.5–20%) was lower and its optimal temperature somewhat higher (50° C) than that of the red-pigmented organisms.

Microscopical Observations

Red-pigmented Rods

At all temperatures up to 45°, as the salt concentration of the medium decreased, cells of the three red-pigmented rods changed from slender, regular, and uniform rods, through a variety of irregular shapes, to almost spherical forms (Fig. 4: A–D, *H. salinarium*; F and G, *H. cutirubrum*; E and H, *H.*

halobium). These changes in morphology were somewhat similar to those noted when cells of *H. cutirubrum* were placed in different salt concentrations (1). As a rule the distorted shapes at the lower salt concentrations tended to be replaced by more rod-like organisms as the incubation time increased.

At any one salt concentration and incubation time the temperature of incubation had little effect on morphology (Fig. 4 C, J, K). At 50°, although there was a definite increase in the turbidity of cultures, cells were not normal; *H. cutirubrum* produced very irregular forms (Fig. 4 L); *H. salinarium* produced a variety of shapes, including long, thick rods (Fig. 4 O, P); and *H. halobium* produced mostly very long thick rods (Fig. 4 M) many of which were bent (Fig. 4 R). With the last organism, many ghosts of the large rods were seen after 90 hours.

The number of inclusion bodies in *H. halobium* increased with age, particularly at high salt concentrations (Fig. 4 H, N, S), and these bodies were more apparent at the low temperatures of incubation.

One end of many of the red rods resembled a hypodermic needle, having a bevelled appearance when viewed from the side and with a more transparent central area when viewed from the top (Fig. 4 K). The opposite end was generally rounded. This bevel was not very noticeable in the long, slender cells of *H. cutirubrum*, but was quite apparent in cells of *H. salinarium* (Fig. 4 J, K) and of *H. halobium* (Fig. 4 H, M). It was not a constant feature and in a single culture, cells varied from square-ended to sharply bevelled (Fig. 4 J).

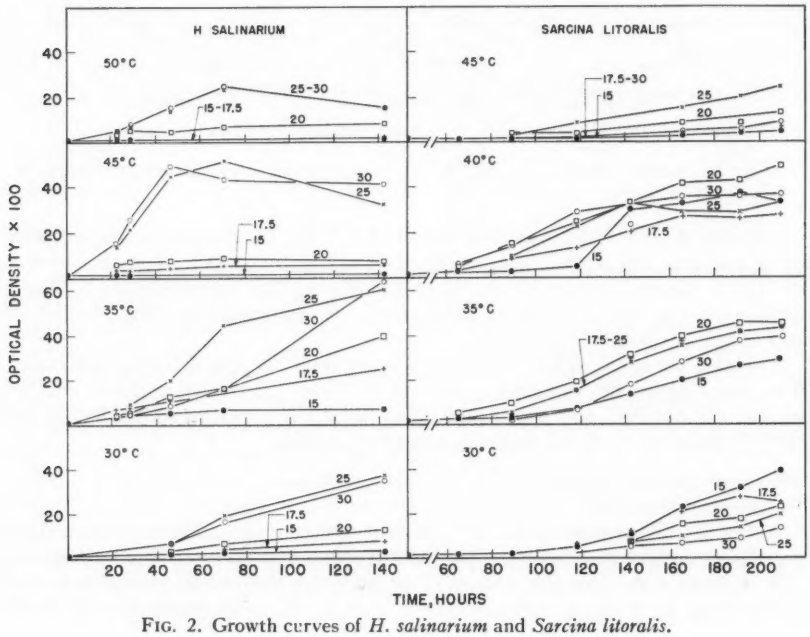


FIG. 2. Growth curves of *H. salinarium* and *Sarcina litoralis*.

Sarcina litoralis

This organism appeared as a coccus, very regular in size and shape at all temperatures and salt concentrations. However, the sarcinal packets and clumps were replaced by pairs and tetrads in all salt concentrations at 30° and 35° C and in 25 and 30% salt at 40°.

Rod A2c

This unpigmented organism grew well at 50° but, unlike the pigmented forms, was a rod at the lower salt concentrations and became more irregular and coccoid with increasing salt concentration. At 55° there were more swollen forms than at the lower temperatures, but high temperatures of incubation affected it less than the red rods.

Discussion

In the experiments reported, temperature intervals were greater than used by some workers, but both temperature and salt concentration were probably controlled more accurately. It was quite evident that at their optimal salt concentrations these organisms grow most rapidly at temperatures between 40° and 50°, depending on the species. At temperatures above the optimum some growth occurred but even in salt concentrations (25–30%) in which normal cells are usually found, the cells were quite abnormal. This seemed to be the only effect of temperature on morphology.

An optimum temperature of about 40° C is generally supported by earlier

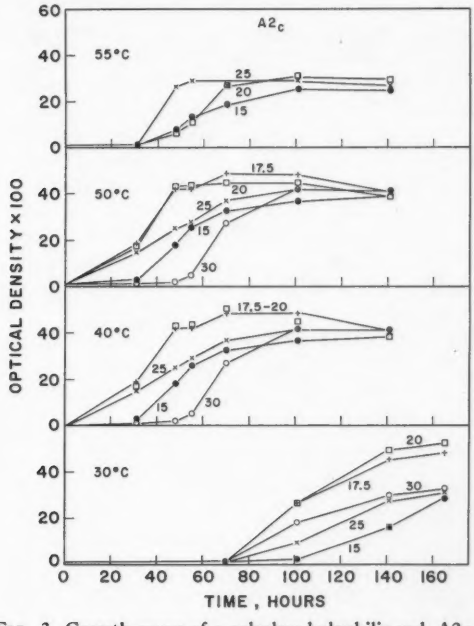


FIG. 3. Growth curve of a colorless halophilic rod, A2*c*.

workers. Harrison and Kennedy (9) stated that *H. salinarium* grew optimally at 42°, that 46° was the maximum temperature, and that no growth was obtained at 48° C. No effect of salt concentration was mentioned, although it was implied that tests were made over a range. Lochhead (11) apparently did not use temperatures over 37° in his studies on *H. salinarium*, *H. cutirubrum*, and *S. litoralis*. Bertullo (2), however, claims that the optimum for *H. salinarium* is 37° and not 42°. Somewhat indirect support for our findings is the observation of Castel and Mapplebeck (6) that *H. salinarium* survived longer in 20% salt solutions at 40° than at 45°, and in 15% salt longer at 25° than at 37°.

Christian (7) reported the number of days required for visible turbidity of *H. halobium* at different salt concentrations and temperatures of 10–45° C. The results confirm the present findings in that most rapid growth was obtained in 25 and 30% brines at 30, 37, and 45° C (1–2 days). However, no growth was obtained in 20% salt at 45° C, conditions under which we obtained excellent growth. In 15% brine he obtained growth at 20, 25, 30, and 37° C in 11, 7, 4, and 20 days respectively, but not at 45° C, indicating a definite temperature optimum at this salt concentration. There were also optimal salt concentrations at 20° C with growth becoming visible only in 20 and 25% salt (17 and 15 days respectively).

Little has been done on the temperature relations of other halophiles to our knowledge, although Browne (5) reported that the optimum temperature for growth of a red spirochaete and a bacterium from salt fish was 45–55° C, and Clayton and Gibbs (8) suggested that material being examined for halophiles should be incubated "at 37° or at 42° C (optimum growth)".

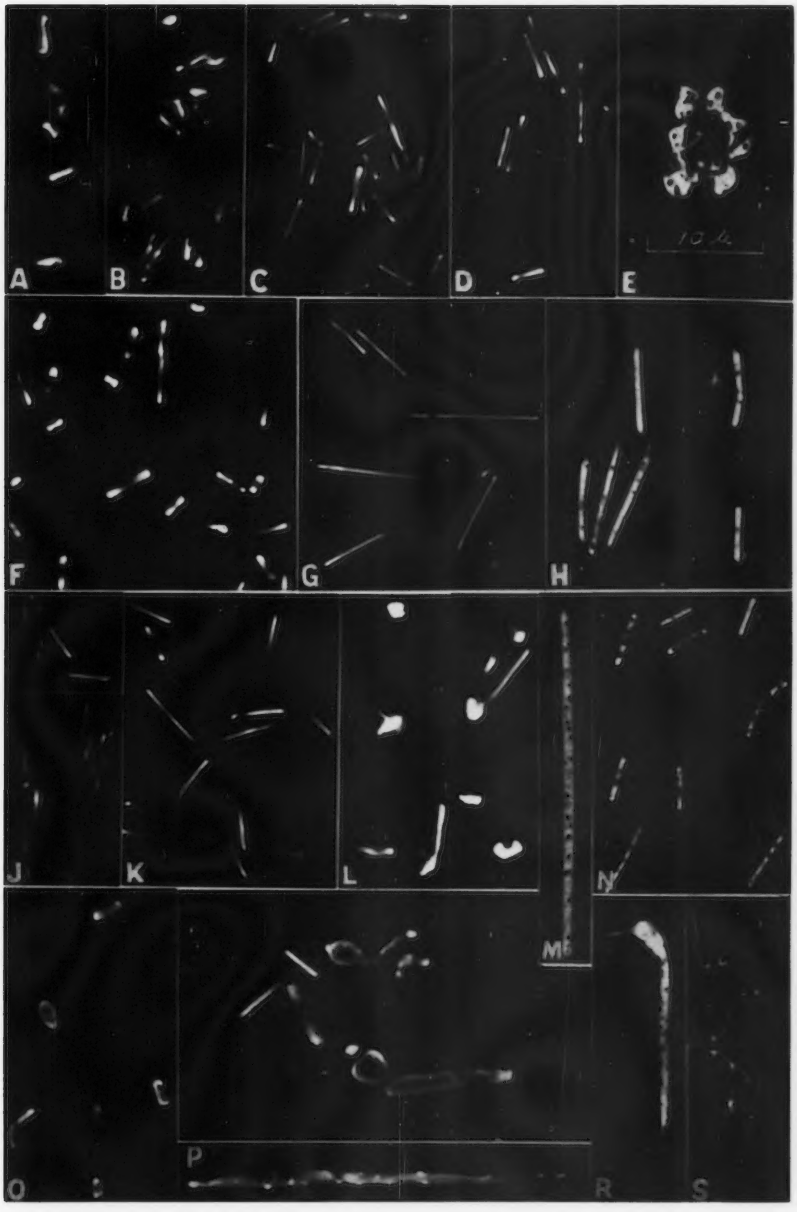
The pleomorphism of the red-pigmented rods has been noted by many workers. Lochhead reported that methanol-fixed cells of *H. salinarium* and *H. cutirubrum* showed greater pleomorphism with age, and that the culture medium, the salt concentration, and the temperature influenced the shape and size of the cells; a slight increase in salt concentration or temperature up to 37° favored the rod form. He also observed that cells of *S. litoralis* increased slightly in size at higher salt concentration, that there was a greater tendency to packet formation at 20–28° than at 37° C, and that sarcina and larger packets were induced by low salt concentration. The present findings confirm these observations in general. They are, however, in direct contrast to the report by Bertullo (3) that cocci predominate in *H. salinarium* at high salt concentrations. The giant ovoid cells noted by this worker are very similar to granule-containing ghosts of forms obtained under what may be called suboptimal conditions; *H. salinarium* normally showed granules, particularly in older cells.

FIG. 4. Photomicrographs of red halophilic rods. Phase contrast, bright H, 1860×. Figures indicate salt concentration in %, temperature of incubation in ° C, and time in hours respectively.

H. salinarium: (A) 17.5%, 35°, 90; (B) 20%, 40°, 65; (C) 25%, 40°, 90; (D) 30%, 40°, 90; (J) 25%, 35°, 90; (K) 25%, 30°, 90; (O) 25%, 50°, 65; (P) 30%, 50°, 65.

H. cutirubrum: (F) 20%, 35°, 167; (G) 20%, 35°, 167; (L) 30%, 50°, 167.

H. halobium: (E) 15%, 35°, 65; (H) 25%, 30°, 65; (M) 30%, 50°, 44; (N) 30%, 35°, 90; (R) 25%, 50°, 44; (S) 30%, 30°, 161.



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The variation in size and shape of these organisms raises the question of whether turbidity is a good measure of growth. However, the change in shape is largely due to change in salt concentration, at least at temperatures up to 45°, so that turbidities at any one salt concentration are comparable. Based on other results it was evident that turbidity gave a very good indication of the relative amount of growth.

Nothing resembling the granular symplasm reported in *H. salinarium* (9, 11) or *H. cutirubrum* (11) had been observed in any of the cultures. Also the large round "gonidangia" and budding coccoid cells reported by others (9, 10, 11) were not seen although spherical forms, especially in older cultures, were readily formed from rods by a variety of means; slight pressure on the cover slip was usually sufficient. It must also be remembered that quite different conditions of growth were used in the various studies mentioned.

As far as can be determined the bevelled ends noted in *H. salinarium* and *H. halobium* have not been mentioned by other workers, although in one of the photomicrographs (Fig. 4 L) of Spruit and Pijper (12) there is a somewhat similar bevel. These authors state that *H. halobium* is ribbon-shaped and if so the hypodermic needle analogy used above would not hold. A bevel could be the result of folding of the ribbon, but in the photographs presented here there is no evidence of such folding and little evidence of a flat shape. When these sluggishly mobile organisms are observed in wet mounts there is considerable evidence of a ribbon-like morphology and further study is required to establish the significance of these morphological observations.

Acknowledgments

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CONTINUOUS CULTURE OF BRUCELLA ABORTUS S.19[1]

ANDREAS H. W. HAUSCHILD[2] AND HILLIARD PIVNICK

Abstract

An apparatus is described for the continuous growth of bacteria. *Brucella abortus* S.19 has been grown in continuous culture for periods up to 3 weeks with populations up to 2×10^{11} viable cells per ml and without the establishment of nonsmooth variants.

Concentrations between 3×10^{9} and 2×10^{11} cells per ml could be maintained as a function of the dilution rate without the requirement of a known limiting factor in the medium. In a series of steady-state conditions, the specific growth rate increased steadily up to 0.28 hour^{-1} with decreasing population levels.

Incidence of mutants was governed by the dilution rate and could also be reduced by various chelating substances.

In continuous growth combined with continuous dialysis, population levels were approximately twice those obtained in continuous growth without dialysis. The effect of dialysis appears to be the continuous removal of growth-limiting metabolic products.

Introduction

Vaccines against infectious abortion of cattle are usually prepared by growth of the moderately virulent strain 19 of *Brucella abortus* on solid media using standard methods established by the United States Department of Agriculture (22), and the Ministry of Agriculture and Fisheries, England (15). However, several reports by van Drimmelen (23, 24, 25, 26) and Sterne (21) have shown that this organism can be grown more economically in liquid culture media by a variety of methods without loss of antigenicity. In our laboratory, attempts have been made to propagate *B. abortus* S.19 by continuous culture in liquid media.

If continuous culture of S.19 for vaccine is to be successful, it is necessary to maintain the original antigenic qualities of the cells throughout the period of continuous growth. Studies of continuous culture of *B. abortus* S.19 and *Brucella suis* have been mentioned briefly by Braun *et al.* (3). They stated that "the gradual establishment of nonsmooth cultures did not occur until 7 days after the start of the continuous culture". This delayed appearance of mutants occurred only when the medium contained antibrucella serum. Without antiserum in the medium mutants developed earlier than 7 days. The publication of Braun *et al.* (3) failed to state the dilution rates used, and, beyond the statement given above, furnished no quantitative data concerning continuous culture.

Gerhardt (10) studied the growth of *B. suis* in continuous culture and found that "strain mutation was minimal after as many as 63 serial transfers". He stated, however, that no virulence tests were conducted and cautioned that strain mutation with respect to virulence might possibly arise. From the foregoing literature we may conclude that continuous culture of *Brucella*

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species is possible, but little is known about antigenic change in continuous culture.

Mutation of *Brucella* species in batch culture has been the subject of numerous studies. These studies have shown that while the presence of cations such as magnesium, manganese (4, 5), and iron (27) and the accumulation of D-alanine (12) are important factors in the selective growth of nonsmooth mutants, the most important single factor is anoxia. In the absence of adequate aeration there is a rapid development of rough mutants while with adequate aeration, cultures remain smooth for extended periods of time regardless of the cations present (1, 2, 19, 21). Thus, with adequate aeration, van Drimmelen (23) and Sterne (21) were able to produce large batches of S.19 suitable for vaccine.

The studies reported in this paper confirm the importance of oxygen in maintaining smooth cultures of *B. abortus*. They also show that the continuous culture of *B. abortus* S.19 may be carried out for extended periods of time without the establishment of nonsmooth mutants. These studies emphasize, however, that the genetic qualities and yield of cells in continuous culture are governed by the dilution rate.

Materials and Methods

Cultures

Brucella abortus strain 19 was obtained from the United States Department of Agriculture and maintained on potato agar slants at 5° C. At intervals of approximately six months a fresh culture was obtained. For production of inoculum for various experiments the culture was grown on solid media similar to those described by the United States Department of Agriculture (22) and washed off with 0.006 *M* phosphate buffer in 0.85% saline at pH 6.4. The suspension was diluted to approximately 200×10^9 viable cells per ml. For each experiment, a fresh preparation of inoculum was used.

Media

The liquid growth medium used was a modification of that described by van Drimmelen (25). It contained: 3% Bacto-Tryptose, 2% glucose (3% glucose was used in studies of continuous dialysis), 1% Marmite (trade name for yeast extract paste supplied by Marmite Ltd., Seething Lane, London, E.C. 3, England), 0.15% Na_2HPO_4, and 0.1% $NaH_2PO_4.H_2O$. The pH was adjusted to 6.6 – 6.8 unless otherwise stated. This medium was sterilized by filtration through asbestos pads because heat sterilization materially reduced cell yields. When antifoam was required, compound AF 60 (8) obtained from the Canadian General Electric Co., Toronto, was employed. This antifoam permitted higher cell yields than any of the several others tested.

The medium used for determining the concentration of viable cells and mutants was Difco Tryptose Agar to which, after autoclaving, 10% of filter-sterilized horse serum was added.

The diluent used for dilution of samples prior to making plate counts consisted of 0.1% Tryptose in 0.5% sodium chloride.

Determination of Cell Concentrations

Cell concentrations were determined by optical density measurements and

plate counts. Optical density was determined at 650 mμ on a Coleman Junior Spectrophotometer, Model 6B (Coleman Instruments, Inc., Maywood, Illinois, U.S.A.) (9). Viable cell concentrations were determined by spreading 0.1 ml of the bacterial suspension on Tryptose horse-serum agar plates in triplicate to obtain between 150 and 250 well-separated colonies. Colonies were counted after 3 days of incubation at 37° C. Several curves relating the optical density measurements of diluted cell suspension to viable counts were obtained and used to expedite various phases of the work as described later. However, optical density measurements were always confirmed by plate counts.

Determination of Mutants

Mutant colonies were determined on the plates used for obtaining viable cell concentrations. The oblique transmitted light method of Henry (13) was first used but discontinued in favor of the staining method of White and Wilson (28). The latter was modified slightly to obtain a staining period of 20 seconds. Plates were examined on the Quebec colony counter (American Optical Co., Buffalo, N.Y., U.S.A.). Two types of variants were observed, rough and mucoid forms. Mucoid colonies never exceeded 4% of the total mutant colonies in any of the experiments described.

Growth of Cultures

All cultures were incubated at 37° C. Static cultures were incubated in 16×125-mm test tubes containing 5 ml of nonaerated medium and shake cultures in 500-ml Erlenmeyer flasks usually containing 80 ml of medium and oscillating at 120 strokes per minute. Continuous cultures were grown in a continuous growth apparatus. Aeration was at 500 ml per minute which gave an oxygen absorption (OAR) of 31 ± 5 mmoles O_2/l./hour as measured by the method of Ecker and Lockhart (7). It was found that at this rate an excess of oxygen was supplied, even to cultures with high bacterial concentrations, because sparging at 500 ml per minute with pure oxygen did not change cell yields although it increased the OAR five-fold. However, a vigorous aeration was required to prevent clogging of the air spargers.

The dilution rate, D, of a continuous culture is defined as the number of complete volume changes per unit of time

$$D = \frac{1}{V}\frac{dV}{dt}$$

and the specific growth rate, μ, is defined by the equation

$$\mu = \frac{1}{X}\frac{dX}{dt},$$

where V is the volume of culture medium in the growth chamber and X is the concentration of organisms. Under steady conditions of continuous growth, where the microbial population in the chamber remains constant, the specific growth rate μ is equal to the dilution rate (14, 16, 17, 18).

Continuous Growth Apparatus

The chemostat described by Novick and Szilard (16) was modified as dis-

cussed below. The continuous dialysis system was used in only a few experiments and, unless otherwise stated, the experiments described in this report were conducted without dialysis. This chemostat suffers from the disadvantage that considerable care is required to adjust the flow rate of medium into the growth chamber but has the advantage of simplicity in design, low cost, and safe handling. During a 10-month period in which over 60 individual chemostat runs were made, often with three to five units operating simultaneously, no contamination occurred.

The apparatus is shown in Fig. 1. Reservoir bottle B and growth chamber C with their attachments are sterilized as two separate units, and the three main components are then connected through ground glass joints G_1 and G_2. Part of the connecting lines D and E is made of rubber tubing to allow for a

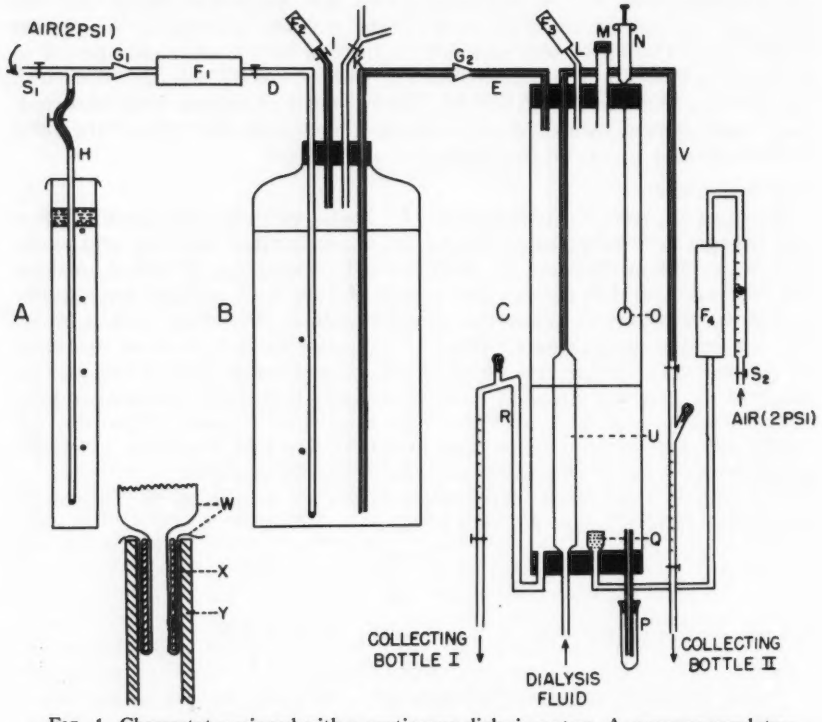

FIG. 1. Chemostat equipped with a continuous dialysis system. A, pressure regulator; B, reservoir; C, growth chamber; D, air line to reservoir; E, medium line; F, filters filled with nonabsorbent cotton (F_1 and F_3, 4×27 cm; F_2, 2×10 cm; F_4, 5×60 cm); G, ground glass joints; H, pressure regulator tube; I, air escape with adjusting screw; K, port for addition of medium, with several female glass joint inlets; L, air escape; M, inoculation port (screw cap test tube with bottom removed); N, antifoam reservoir; O, antifoam screen; P, sampling device; Q, fritted air sparger (coarse, 12 mm diameter); R, outflow; S, air line from pressure tank; T, air flow meter (Fischer & Porter Co., Toronto, Canada, range 96–960 ml/min); U, dialysis tube; V, outflow from dialysis tube; W, cellophane tubing; X, glass tubing; Y, rubber tubing.

high degree of flexibility. Pressure regulator A, 60 cm long and 5 cm in diameter, is filled with water and a layer of mineral oil, and covered with aluminum foil to reduce evaporation. B, a 10-liter pyrex bottle, is filled through K, which is branched to allow several additions of medium, each through a fresh ground glass joint. Growth chamber C is a pyrex tube identical with A in size and closed at each end with a rubber stopper. Each stopper is held in place by a flat metal ring having inside and outside diameters of 4 and 8 cm respectively. These metal rings, at their outer rim, have two diametrically opposite holes through which pass two stainless steel clamping rods. Adjusting wing nuts on the ends of the metal rods creates pressure on the stoppers and makes a tight seal. Antifoam reservoir N is a 2- or 5-ml syringe. This is attached to a Luer-lock needle butt to which is welded 38 cm of No. 14 gauge stainless steel tubing. To the lower end of the tubing is fastened a disk, 20 to 25 mm in diameter, of 100-mesh stainless steel screen which holds sufficient antifoam to control foaming while the apparatus is not being attended. The screen is held about 6 cm above the level of the culture (volume of culture is 250 ml). Additions of antifoam are made by turning the screw of a metal pipetting holder which encloses the syringe. The sampling device consists of a capillary tube which is sealed off by a test tube filled with 95% alcohol. The capillary tube is interrupted by a small piece of rubber so that the line can be clamped off. The liquid level in the growth chamber is set by the height of the outgoing tube R, for which a wide (11 mm I.D.) diameter is required. A second reservoir bottle assembled like B but holding 20 liters of medium is attached to the air pressure tank via a filter identical with F_1 on one side, and to the dialysis system through the outgoing line. The cellophane bag W (19 mm diameter) extends through the medium of the growth chamber. It is held in place by pulling it through a 5-cm-long glass tubing, X (7 mm inside diameter), folding it back on the outside, slipping rubber tubing Y over the cellophane, and wiring the attachment. The media inside and outside the dialysis tube are identical.

Operation of the Apparatus

With H and the connecting lines D and E open, continuous flow is started by adjusting the air flow to allow air to bubble slowly through A and, to a lesser extent, through B. The exact flow of medium is adjusted by raising and lowering H in A and measured by relating the number of drops per time unit to the volume collected in a calibrated portion of R.

The medium in the second reservoir bottle is forced through the dialysis system and the flow rate measured as described above. The flow rate is adjusted by a screw clamp between the outgoing line V and collecting bottle II.

To start a continuous growth experiment, the growth chamber of the chemostat was filled to the 250-ml mark with medium from the reservoir, antifoam was added in minimal amounts, and aeration started at 500 ml per minute. Aeration was continued overnight to test sterility, and growth was then initiated by introducing inoculum sufficient to obtain 2 to 4×10^9 cells per ml of medium. The culture remained undiluted until the approximate desired bacterial populations were obtained as measured by optical density methods. At this stage continuous flow of fresh medium was started and maintained

at a constant flow rate. Within 24 hours bacterial densities usually became constant.

Results

Effect of the Dilution Rates on Bacterial Concentrations During Continuous Growth of Brucella abortus S.19

Four chemostats were run simultaneously starting with low dilution rates. Bacterial concentrations were determined when at least two successive samples taken at intervals of 4 hours or more were identical with respect to optical density. The dilution rates were then successively increased. The reverse procedure, that is starting at high dilution rates, was not feasible because of the rapid establishment of nonsmooth variants (see Fig. 6). Experiments were discontinued whenever the mutants exceeded 5% of the viable cells.

Figure 2 shows that the maximum cell yield per hour was obtained at $D = 0.083$ hour^{-1} corresponding to a complete change of medium in the growth chamber within 12 hours.

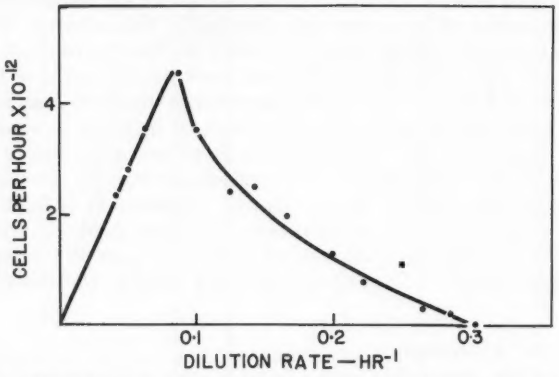

FIG. 2. Effect of dilution rate on cell yield per hour of continuous culture.

Figure 3 shows the effect of the dilution rate on the concentration of viable bacteria. Note that the highest concentrations were obtained at dilution rates between 0.08 hour^{-1} and 0.04 hour^{-1}. These correspond to complete replacements of the medium in the growth chamber in approximately 12 and 24 hours respectively. With increasing dilution rates, lower bacterial concentrations were maintained at a steady state. Since under the above conditions D equals μ, it can be concluded that with decreasing bacterial concentrations the specific growth rate steadily increased up to $\mu = 0.28$ hour^{-1} (Fig. 3).

The relationship between bacterial concentrations and dilution rates was also found in another experiment (Fig. 4). In addition, Fig. 4 demonstrates that even at high dilution rates bacterial concentrations could be held relatively constant over several days.

The result suggests that *B. abortus* may not exhibit logarithmic growth at concentrations exceeding 3×10^9 cells per ml. However, several growth

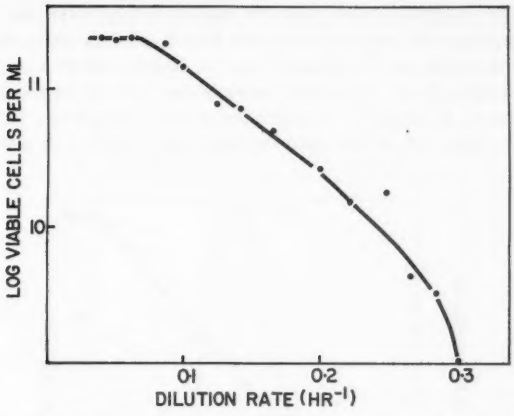

FIG. 3. Effect of the dilution rate on bacterial concentrations at steady state of continuous growth.

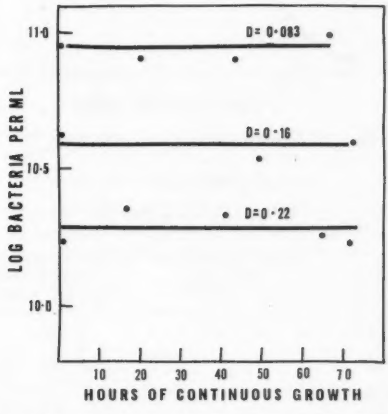

FIG. 4. Constancy of viable populations of *B. abortus* S.19 at different dilution rates.

curves (e.g., Fig. 5) failed to show any deviation from logarithmic growth until bacterial concentrations were at least 30×10^9 cells per ml or higher.

During the experiments depicted in Fig. 3 it was found that the pH of the culture varied between 6.6 for the uninoculated medium and 7.6 at the lowest dilution rate ($D = 0.04$ hour^{-1}) investigated. To test whether these changes accounted for the observed increase in specific growth rate with decreasing bacterial concentration, *B. abortus* was grown in batch culture in triplicate at different pH. The results, as shown in Table I, demonstrate that the growth rate is not markedly affected by the above changes in pH.

It was found that different batches of Tryptose yielded different bacterial concentrations. For example, in two chemostats treated identically except

for the batch of Tryptose used in the medium, concentrations of 212×10^9 and 80×10^9 bacteria per ml were obtained at a dilution rate of 0.083 hour^{-1}. With three other lots of Tryptose used in batch culture experiments, cell counts varied from 58 to 177×10^9 bacteria per ml. It was suggested by the Digestive Ferment Company, manufacturers of Tryptose, that when large amounts of Tryptose are to be used, preliminary testing of a small sample is desirable.

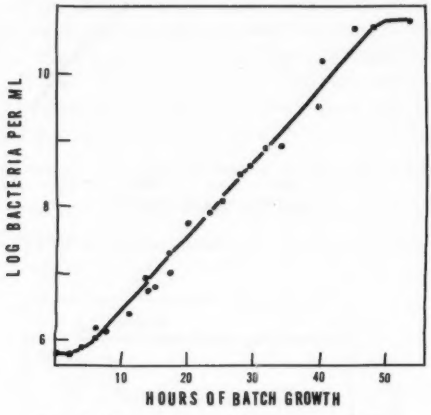

FIG. 5. Growth curve of *B. abortus* S.19.

TABLE I

Effect of pH on growth

Initial pH	pH after 7 hours	Initial population (viable cells/ml $\times 10^{-8}$)	Population after 7 hours (viable cells/ml $\times 10^{-8}$)	Mean generation time in hours
7.2	7.4	5	27	2.9
6.8	7.0	5	26	2.9
6.5	6.7	5	24	3.1

Establishment of Nonsmooth Variants During Continuous Growth

During our studies described above it was observed that nonsmooth variants became established readily in originally smooth cultures at high dilution rates, while at low dilution rates the cultures remained virtually unchanged over extended periods of time. The percentage of rough and mucoid variants rose to 40–70% within a week when the medium was replaced within 4 hours (D = 0.25 hour^{-1}), but did not exceed 0.2% within 3 weeks when the time of complete replacement was 12 hours (D = 0.083 hour^{-1}) or higher. Figure 6 shows that the establishment of mutants could be reversed by changing from high to low dilution rates. It was shown by pH determinations that due to changes in bacterial concentrations the pH increased from 7.0 to 8.0 when the dilution rate was decreased from 0.25 hour^{-1} to 0.04 hour^{-1}.

The rapid rise and decline in the percentage of variants (Fig. 6) appears to be caused by differential growth rates of rough and smooth cells. This is confirmed in the following experiment, in which specific growth rates of the original smooth strain and of a rough variant were determined as a function

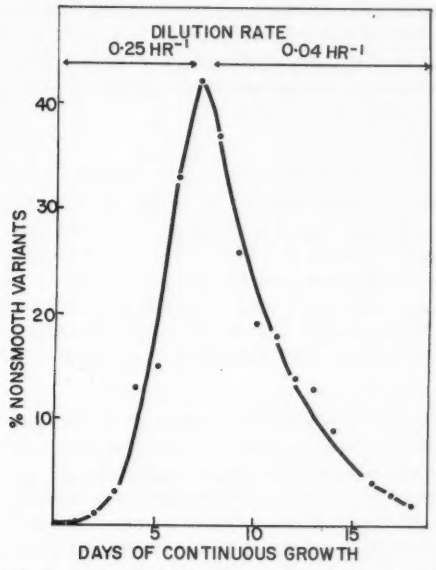

FIG. 6. Effect of dilution rate on selection of nonsmooth mutants of S.19 in continuous culture.

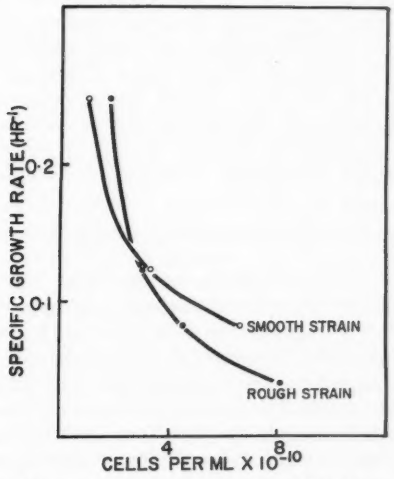

FIG. 7. Growth rates of rough and smooth strains of S.19 in continuous culture.

of bacterial cell concentrations in continuous culture (Fig. 7). Note that the specific growth rate of the rough variant exceeded that of the smooth form in low bacterial concentrations but decreased more rapidly with increasing cell concentrations.

The observed population changes at different dilution rates may have been caused by one or more of the following factors: 1, pH; 2, CO_2 concentration; 3, a factor present in the fresh medium; 4, a factor other than CO_2 being released by the organisms. To test some of these hypotheses, several experiments were carried out.

Effect of pH

Two chemostats, A and B, were run with dilution rates adjusted to 0.25 $hour^{-1}$ and 0.04 $hour^{-1}$ respectively. In order to maintain comparable pH values, the supply medium for A was adjusted to pH 7.6 and that for B to pH 6.5. In continuous culture, the pH in A was 7.8 and in B 7.6.

Within 7 days of continuous growth, the percentage of nonsmooth variants increased from 0% to 45% in A and from 0% to 0.2% in B. It is concluded that in the range between pH 7 and pH 8 the hydrogen ion concentration has no decisive effect on the establishment of mutants.

CO_2 Concentration

Cultures with high and low dilution rates, equally aerated, differ in bacterial concentrations and, therefore, in concentration of CO_2 produced by metabolic activity. It is expected that at high dilution rates and consequent low cell densities the CO_2 content of the culture would be relatively low. To test the effect of increased CO_2 concentration, cultures with high dilution rates (D = 0.25 $hour^{-1}$) were aerated with air containing 5% CO_2. Addition of CO_2, however, did not curtail the emergence of mutants.

A Factor in the Fresh Medium

Several factors in the medium may be of importance in the selective growth of nonsmooth mutants at high dilution rates. Schuhardt *et al.* (20) found that elemental sulphur in Tryptose inhibited growth of many but not all strains of *Brucella* species tested. Cole and Braun (5) and Cole (4) showed that the bivalent cations of manganese and magnesium promoted the growth of nonsmooth mutants of *Brucella*. Waring *et al.* (27) found that iron selectively promoted the growth of nonsmooth mutants of *B. suis* and that an iron-deficient medium tended to maintain test tube cultures in the smooth form. However, Waring *et al.* failed to confirm Cole's findings with magnesium and manganese.

In our experiments, treatment of the medium with Norite A (20) to remove elemental sulphur did not suppress the selective growth of mutants when the media were replaced in 4 hours (D = 0.25 $hour^{-1}$). On the contrary, the percentage of mutants rose to 91% within 1 week in the treated medium compared with 45% in the untreated medium. Apparently, elemental sulphur does not select in favor of the nonsmooth mutants at high dilution rates.

In the following experiments the effect of inorganic cations was tested. In order to reduce the concentration of free magnesium, manganese, iron, and other cations, media were treated with various chelating agents. Experi-

mental conditions and the results are given in Table II. Note that the three chelating agents tested suppressed the establishment of nonsmooth variants. The suppressing effect was more pronounced with batch 1 than with batch 2 of Tryptose.

TABLE II

Effect of metal complexing compounds on the establishment of nonsmooth variants in continuous cultures of *Brucella abortus* S.19 with high dilution rates (D = 0.25 hour^{-1})

Tryptose batch	Metal complexing compound	Concn. (molarity)	Average bacterial concn. (viable cells/ml $\times 10^{-9}$)	% mutants after 7 days' cont. growth
I	None	—	4.1	45
	None	—	7.2	42
	Sodium citrate	5×10^{-3}	5.0	7
	Versene	1.5×10^{-4}	6.4	5
II	None	—	8.6	34
	Sodium citrate	6×10^{-3}	8.7	12.5
	Versene	3×10^{-4}	7.9	19
	Sodium pyrophosphate	6×10^{-3}	5.9	11.5

The pronounced effect of chelation on the suppression of mutants at high dilution rates (and concomitant low bacterial concentrations) suggested that at low dilution rates (and concomitant high bacterial concentrations) the bacterial mass might be synthesizing into cell material most of the cations required for growth of nonsmooth mutants. This would then explain the failure of nonsmooth mutants to develop in continuous cultures run at dilution rates of 0.08 hour^{-1} and 0.04 hour^{-1}. To test this, 13 bivalent cations of Mn, Pb, Sn, Cu, Zn, Cd, Mg, Co, Sr, Ba, Co, Ni, Fe, and the trivalent cations of Fe and La were supplied to the medium in concentrations between 10^{-5} and 3×10^{-3} M. Six chemostats were run, and salts were added in such combinations that would not give rise to any precipitation in water. However, some precipitations from cations and ingredients of the medium could not be avoided. The dilution rate was maintained at 0.04 hour^{-1} and the percentage of nonsmooth variants determined over a period of 12 days. It was found that in none of the cultures did the variants exceed 1%. The effect of chelates and the role of a cation in the establishment of mutants are yet to be determined.

Clone Selection

In an attempt to select cultures with the least tendency to dissociate as a source of inoculum for continuous growth, 22 clones derived from a smooth culture were incubated under static conditions in test tubes at 37° C. In substantiation of the results of Braun (2) it was found that after 6 days the percentage of mutants in the samples varied from 3% to 98%. Two groups of four clones each showing 1–10% and 95–100% variants respectively were selected and inoculated in their original smooth form into shake flasks. It was found that over a period of 9 days the percentage of nonsmooth variants in all cultures remained below 1%. Since shake flask conditions resemble those in continuous growth at low dilution rates, the selection of clones was considered to be of no advantage for the production of smooth cultures.

Effect of Continuous Dialysis

Dialysis is of value in producing large viable populations of *B. abortus* S.19. Sterne (21) grew S.19 inside an aerated dialysis tube on a batch basis but with nutrients supplied by continuous flow of fresh medium around the membrane. He introduced into the dialysis tube only saline and bacteria, and thus the nutrients were supplied only by dialysis. After 3 to 4 days viable populations of bacteria averaged about 800×10^9 cells per ml.

In our experiments, S.19 was grown in the usual medium outside the dialysis tube (Fig. 1) with a dilution rate of 0.04 $hour^{-1}$. The medium within the tube was added continuously at a rate of 500 ml per day. Two experiments were run, each for several days, and each with a different lot of Tryptose in the medium. As controls, chemostats without dialysis were run using the same media, dilution rates, and aeration. The results are shown in Table III.

TABLE III

Effect of continuous growth with continuous dialysis

	Viable bacteria per ml $\times 10^{-9}$	
Tryptose batch	With dialysis	Without dialysis
I	375	152
II	80	38

Increased populations with continuous dialysis appear to be caused by one or more of the following factors: (1) continuous supply of nutrients; (2) the retention of inhibitory materials outside the dialysis membrane (in Sterne's experiments); and (3) the removal of toxic products of metabolism.

The effect of nutrient supply was tested in the following experiment: 500-ml Erlenmeyer flasks containing 50 ml of either regular medium or regular medium diluted 1:1 with distilled water were inoculated to furnish 5×10^9 cells per ml. After 23 hours of growth both flasks contained 100×10^9 cells per ml. These results indicate that all components of the medium were in excess and that increases in yield due to continuous dialysis were probably gained by a factor other than the additional supply of nutrients.

The following experiment was conducted to determine whether non-dialyzable products in the medium might limit the growth of *B. abortus*. Double strength medium was introduced aseptically into a flask along with a dialysis sack containing an equal volume of distilled water. An identical system, but with the dialysis bag punctured, was used as a control. After 2 days of slow

TABLE IV

Growth of *B. abortus* in dialyzed and nondialyzed media

	Viable cells per ml $\times 10^{-9}$ after growth for (hours):					
	0	5	12	17	22	27
Dialyzed medium	1.3	2.4	12.8	35	58	73
Nondialyzed medium	1.3	2.3	12.3	32	61	75

shaking at 22° C, to allow for equilibration through the membrane, 30-ml amounts of media were transferred from the dialysis sack and from the control flask to flasks which were inoculated and shaken. Table IV shows that there was no difference in growth between the two media. It may be concluded that dialysis does not serve to retain inhibitory products but appears to function by a continuous removal of growth-inhibiting by-products of metabolism.

Discussion

The results of these studies indicate that continuous growth of *B. abortus* S.19 in high yield and without apparent mutation is possible. The economy of production of S.19 vaccine in liquid culture has been emphasized by both van Drimmelen and Sterne. Further economies may be expected with continuous culture.

With increasing dilution rates, bacterial concentrations decreased but were held constant up to a dilution rate of $D = 0.28$ hour^{-1} corresponding to a bacterial concentration of 3×10^9 cells per ml. At steady conditions of continuous growth the dilution rate D is equal to the specific growth rate μ (14). Therefore, the specific growth rate steadily increased with decreasing bacterial concentrations down to 3×10^9 cells per ml. The result suggests that the logarithmic phase in batch cultures should not extend beyond the above cell concentration. However, logarithmic growth of *B. abortus* usually continued up to 30×10^9 cells per ml. The discrepancy between the findings still remains to be explained.

Generally, one of the prerequisites for the maintenance of steady-state conditions in cultures with bacterial populations below maximum is the presence of a controlling growth factor (14, 16). The fact that despite the lack of such a factor steady-state conditions could be maintained in cultures of *B. abortus* over a wide range of dilution rates might be explained by the secretion of autotoxic materials from the bacterial cells. These substances would increase in concentration with rising bacterial concentrations, thus establishing a definite relation between cell concentration and specific growth rate. The experiments on the effect of dialysis indicate the existence of such materials in cultures of *B. abortus*. It should be mentioned that Contois (6) has shown that in the continuous culture of *Aerobacter* the specific growth rate is also a function of the cell density.

It was observed that at high dilution rates nonsmooth mutants were readily established while at low dilution rates and concomitant high bacterial concentrations the cultures remained virtually unchanged. This observation was explained by determinations of specific growth rates, which showed that at high bacterial concentrations the smooth strain grows at a faster rate than a rough mutant but grows at a slower rate than the variant at low bacterial concentrations. The result is contrary to the work of Altenbern *et al.* (1), who found that growth rates of rough variants were below those of a smooth strain in cultures with low as well as high bacterial concentrations. The different results may be due to the particular medium employed (11), to the isolation of different rough variants, or to the fact that Altenbern *et al.* used batch culture while our experiments were conducted with continuous culture.

Differences in pH and CO_2 concentration between cultures with high and

low cell concentrations appeared to have no decisive effect on the establishment of nonsmooth variants. However, metal-complexing substances caused a definite delay in the establishment of mutants. Although addition of various cations to cultures at a high dilution rate failed to increase the percentage of mutants substantially, the observed effect of chelates suggests that some cation is involved in establishment of nonsmooth mutants.

When continuous growth was combined with continuous dialysis, bacterial concentrations could be doubled. It was found that the effect of dialysis is due neither to an additional supply of nutrients nor to the retention of growth-inhibiting substances by the cellophane bag outside the culture. By eliminating the above factors it is concluded that the effect of dialysis is exerted by removal of autotoxic substances from the culture of *B. abortus*.

Before the method of continuous growth may be used for vaccine production, several additional problems must be considered. The apparent lack of mutation, as manifested by appearance of colonies on agar, may not be real. Smooth colony strains may contain cells which are completely avirulent or extremely virulent and, therefore, examination of colonies is not sufficient proof of suitability of continuously grown S.19 for vaccine. Indeed, S.19 is a strain of intermediate virulence. For this reason continuously grown S.19 must be tested in animals. Such experiments have been undertaken and the results will be presented in a later publication.

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STUDIES ON THE INTERACTION OF PLANTS AND FREE-LIVING NITROGEN-FIXING MICROORGANISMS

II. DEVELOPMENT OF ANTAGONISTS OF AZOTOBACTER IN THE RHIZOSPHERE OF PLANTS AT DIFFERENT STAGES OF GROWTH IN TWO SOILS[1]

E. STRZELCZYK[2]

Abstract

This study represents an attempt to correlate the low numbers of *Azotobacter* in rhizosphere and root-free soils at the Central Experimental Farm, Ottawa, with the incidence of bacterial and actinomycete antagonists of this organism. Wheat, radish, and onion were grown in the greenhouse in two soils varying greatly in fertility and organic matter content, and isolations of bacteria and actinomycetes were made periodically for testing against *Azotobacter*. It was found that rhizosphere soil contained greater numbers of microorganisms antagonistic to *Azotobacter* than root-free soil. Of the three crops used wheat exerted the least effect. In all the tests numbers of antagonists were greater in the fertile Granby sandy loam than in the infertile Upland sand. The results correlated well with the *Azotobacter* populations in these soils as reported in the first paper of this series.

Introduction

In the first paper of this series (7) it was reported that numbers of *Azotobacter* were extremely low in the rhizosphere of 17 crop plants and in the adjacent nonrhizosphere soil. Counts varied with the age and type of plant and with the kind of soil used. Buckwheat showed the greatest rhizosphere effect on these bacteria (rhizosphere:soil ratio of 17.8) whereas, tobacco, mustard, and flax had no effect. Although greenhouse experiments showed improved growth of *Azotobacter*, especially in the rhizosphere of wheat, the counts were still very low in comparison with those of other soil bacteria and it was suggested that *Azotobacter* could not compete successfully with other soil microorganisms. A number of investigators have reported the inhibition of *Azotobacter* by bacteria and actinomycetes in the rhizosphere and in soil devoid of roots (1, 5, 13, 20, 22), and it was considered important to investigate this phenomenon in greater detail in an effort to explain the low counts obtained. The production of inhibitory substances by plant roots, as shown by Bukatsch (2) and Metz (11), is considered a special case which probably does not apply to most crop plants. Since other factors such as soil fertility and reaction are also known to affect *Azotobacter* (6), two soils varying greatly in pH and fertility were used. The effect of kind of plant and stage of growth in relation to the development of *Azotobacter* antagonists was also investigated.

Materials and Methods

Wheat, radish, and onion were grown in the greenhouse in soils varying markedly in fertility and in organic matter content (7). Bacterial and actino-

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mycete antagonists of *Azotobacter* were obtained by plating rhizosphere and nonrhizosphere soil samples with soil extract agar and isolating all the colonies on a plate or representative portion thereof after incubation for 14 days at 28° C. The bacterial colonies were inoculated into soil extract semisolid agar. After 5 days' incubation at 28° C the cultures were transferred to Petri plates containing the following medium: glucose, 5.0 g; KNO_3, 1.0 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; NaCl, 0.2 g; $FeSO_4$, trace; Antibiotic Medium 2 (Difco), 2.0 g; yeast extract (Difco), 0.8 g; agar, 15.0 g; soil extract, 200 ml; H_2O (distilled), 800 ml; pH 7.0. Actinomycetes were picked and transferred to slopes and later to Petri dishes poured with the following medium: glucose, 5.0 g; KNO_3, 1.0 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; $FeSO_4$, trace; agar, 15.0 g; H_2O (distilled), 1000 ml; pH 7.0. After 7 days' incubation at 28° C agar disks were cut from the bacterial and actinomycete growth and transferred to the surface of nitrogen-free sucrose agar seeded with *Azotobacter chroococcum*. Plates were kept for 24 hours at room temperature to permit diffusion of inhibitory substances from the agar disks into the *Azotobacter* medium. After 48 hours' incubation at 28° C the width of the inhibition zone surrounding the agar disks was measured.

Results

The actinomycete and bacterial counts in rhizosphere and root-free soil (Tables I and II) show the usual rhizosphere effect—an increase in numbers in the root zone. This is less for actinomycetes (Table I) than for bacteria (Table II) as reported also in most rhizosphere studies (8, 15). In general it appears that the rhizosphere:soil ratio (R:C) for actinomycetes is higher in Granby sandy loam than in Upland soil, whereas the reverse effect may be observed with bacteria. In addition, it is clear (Fig. 1) that the rhizosphere contains greater numbers of microorganisms (actinomycetes and bacteria) antagonistic to *Azotobacter*. The variation in number of antagonists is not large in the control soil but in the rhizosphere considerable fluctuation may be noted due to the kind and age of the plant and to the type of soil used. Wheat shows the least influence on the number of antagonists whereas both onion and radish exert a significant positive effect. In every case rhizosphere soil and control soil counts of antagonists are higher in the Granby sandy loam.

Actinomycetes Antagonistic to Azotobacter

Although the percentage incidence of antagonistic actinomycetes is generally greater in nonrhizosphere soil, absolute numbers are higher in the rhizosphere as attested by the rhizosphere:soil ratios in the last column of Table I. By far the greatest proportion of these antagonists in the control soil produced very small zones of inhibition (zone of 1–5 mm). On the other hand, the majority of the cultures showing inhibition zones of 6–10 mm or >10 mm occurred in the rhizosphere. The most striking plant effect was obtained with onion with both soils, especially at 100 days. The Granby sandy loam contained more antagonists than did the Upland sand.

Bacteria Antagonistic to Azotobacter

In every instance there are larger numbers of bacteria antagonistic to *Azotobacter* in the rhizosphere than in the control soil (Table II). The root

TABLE I

Total numbers of actinomycetes and numbers of isolates antagonistic to *Azotobacter* in rhizosphere and control soil

Type of soil	Kind of plant	Age of plant (days)	Total nos.*			Isolates antagonistic to *Azotobacter*							
						Control				Rhizosphere			
						%				%			
			Control	Rhizosphere	R:C†	Width of zone of inhibition in mm: 1–5	6–10	>10	Total no.* and %‡	Width of zone of inhibition in mm: 1–5	6–10	>10	Total no.* and %‡	R:C
Granby sandy loam	Radish	21	4.4	62.0	14.0	31	8	5	1.90 (44)	5	11	8	15.0 (24)	8.0
		70	1.9	25.0	13.0	30	8	2	0.76 (40)	11	12	5	7.0 (28)	9.0
		100	2.4	24.4	10.0	11	0	3	0.33 (14)	4	8	6	4.3 (18)	13.0
	Onion	21	4.4	11.6	2.5	31	8	5	1.90 (44)	15	7	0	2.6 (22)	1.5
		70	1.9	45.0	23.5	30	8	2	0.76 (40)	7	10	8	11.2 (25)	15.0
		100	2.4	90.7	37.5	11	0	3	0.33 (14)	5	3	4	11.0 (12)	33.0
	Wheat	21	4.4	80.9	18.5	31	8	5	1.90 (44)	5	3	2	8.0 (10)	4.0
		70	1.9	36.8	19.5	30	8	2	0.76 (40)	11	5	3	7.0 (19)	9.0
Upland sand	Radish	21	1.7	28.9	17.0	22	3	1	0.44 (26)	10	6	4	5.8 (20)	13.0
		70	2.8	14.2	5.0	15	3	2	0.56 (20)	9	4	5	2.5 (18)	4.5
		100	0.6	5.3	9.0	14	3	1	0.10 (18)	5	3	1	0.5 (9)	5.0
	Onion	21	1.7	3.5	2.0	22	3	1	0.44 (26)	12	5	4	0.7 (21)	1.5
		70	2.8	30.3	11.0	15	3	2	0.56 (20)	5	4	1	3.0 (10)	5.5
		100	0.6	27.6	46.0	14	3	1	0.10 (18)	4	5	6	4.2 (15)	42.0
	Wheat	21	1.7	15.8	9.0	22	3	1	0.44 (26)	6	6	6	2.7 (18)	6.0
		70	2.8	7.6	2.5	15	3	2	0.56 (20)	7	2	3	0.9 (12)	1.5

*Expressed in millions per gram oven-dry soil.

†$R:C = \frac{\text{Numbers in rhizosphere soil}}{\text{Numbers in control soil}}$.

‡Percentages in parentheses.

TABLE II

Total numbers of bacteria and numbers of isolates antagonistic to *Azotobacter* in rhizosphere and control soil

Type of soil	Kind of plant	Age of plant (days)	Total nos.*			Isolates antagonistic to *Azotobacter*								
						Control				Rhizosphere				
						%				%				
			Control	Rhizosphere	R:C†	Width of zone of inhibition in mm: 1–5	6–10	>10	Total no.* and %‡	Width of zone of inhibition in mm: 1–5	6–10	>10	Total no.* and %‡	R:C
Granby sandy loam	Radish	21	5.6	95.3	17.0	13	4	1	1.0 (18)	11	7	5	23.0 (24)	23
		70	8.7	322.6	37.0	9	5	0	1.2 (14)	8	5	5	58.0 (18)	88
		100	9.4	142.9	15.0	15	2	2	1.8 (19)	10	8	6	34.3 (24)	19
	Onion	21	5.6	21.2	4.0	13	4	1	1.0 (18)	7	8	5	4.2 (20)	4
		70	8.7	256.4	29.5	9	5	0	1.2 (14)	4	3	3	25.6 (10)	21
		100	9.4	587.6	62.5	15	2	2	1.8 (19)	2	6	4	70.5 (12)	39
	Wheat	21	5.6	158.3	28.0	13	4	1	1.0 (18)	5	3	3	17.4 (11)	17
		70	8.7	109.2	12.5	9	5	0	1.2 (14)	2	3	5	16.4 (10)	14
Upland sand	Radish	21	2.8	60.0	21.5	7	3	0	0.28 (10)	12	4	5	12.6 (21)	45
		70	2.0	87.4	43.0	9	2	0	0.22 (11)	3	6	3	10.4 (12)	48
		100	1.4	44.7	32.0	18	4	2	0.33 (24)	5	6	5	7.0 (16)	21
	Onion	21	2.8	16.3	6.0	7	3	0	0.28 (10)	8	6	4	2.9 (18)	10
		70	2.0	94.6	48.0	9	2	0	0.22 (11)	10	5	4	17.9 (19)	81
		100	1.4	117.0	83.5	18	4	2	0.33 (24)	7	3	0	11.7 (10)	35
	Wheat	21	2.8	38.0	13.5	7	3	0	0.28 (10)	10	4	0	5.3 (14)	19
		70	2.0	32.9	17.0	9	2	0	0.22 (11)	8	1	1	3.2 (10)	15

*Expressed in millions per gram oven-dry soil.

†$R:C = \frac{\text{Numbers in rhizosphere soil}}{\text{Numbers in control soil}}$.

‡Percentages in parentheses.

effect is also greater for bacterial than for actinomycete antagonists. The percentage of these bacteria was found to be much higher in both the rhizosphere and control soil in the Granby sandy loam series. Radish and onion rhizospheres appeared to exert greater effect on numbers of antagonists than did wheat in both soils and especially at the 70-day sampling period. Again, as with the actinomycetes, the percentage incidence of bacteria producing zones over 6 mm in size was greater in the rhizosphere than in root-free soil.

Discussion

The abundance of antagonistic actinomycetes and bacteria in the two soils and in the rhizosphere of the three crops studied may be partly responsible for the small number of *Azotobacter* relative to the general microbial population as reported in the first paper of this series (7). It was also reported that the R:C ratios for *Azotobacter* were lowest in the Granby sandy loam; this is particularly interesting since the rhizosphere of the plants grown in this soil contained the greatest number of antagonists (Fig. 1). Furthermore, an inverse relationship is evident between the high R:C ratios of *Azotobacter* on wheat roots (7) and the low number of antagonists in both soils. This is particularly striking in the Upland sand. A similar correlation exists for radish in this soil, i.e. an increase in R:C ratio for *Azotobacter* with time (7) and a concurrent decrease in number of antagonistic organisms (Fig. 1).

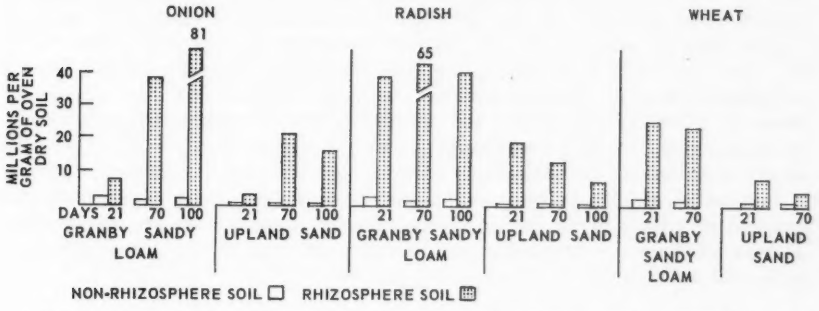

FIG. 1. Numbers of microorganisms (bacteria and actinomycetes) antagonistic to *Azotobacter* in the rhizosphere of plants at different stages of growth in two soils.

The suppression of the nitrogen-fixer by these organisms presupposes the production of inhibitory substances in both rhizosphere and nonrhizosphere soils under natural conditions. There is some evidence that under certain rather artificial conditions (21) antibiotics may be found in nonsterile soil. Stevenson (18, 19), using a sensitive microscopic technique, was able to demonstrate antibiotic effects in normal soil which did not yield detectable amounts of antibiotic by extraction. The evidence is in conformity with the hypothesis that in the root zone conditions are favorable for the production of minute but effective amounts of inhibitory substances. The excretion by plant roots of soluble organic substances or their liberation through autolysis of dead or dying root parts would indeed favor the activity of antagonists

as well as other organisms. Buxton (3) observed, for example, that the presence of pea root exudates enhanced the ability of rhizosphere fungi to inhibit *Fusarium oxysporum* f. *pisi*. The fact that some plants exert a greater effect on numbers of antagonists at certain stages of growth than others in the same soil implies that root excretions or related phenomena may play a very important role. It has also been demonstrated by Krasilnikov (9) that soils rich in organic matter contain larger numbers of antagonistic actinomycetes than poor soils. In the present study, Granby sandy loam with 5% organic matter contains larger numbers of *Azotobacter* antagonists than the very poor Upland sand with 1.2% organic matter (7). Yugina (22) found that the degree of toxicity of cultivated as well as noncultivated soils to *Azotobacter* depends often on the presence of antagonistic organisms in these soils.

Although the correlation between abundance of *Azotobacter* antagonists and paucity of *Azotobacter* appears strong, the factor of competition cannot be dismissed. A number of workers (10, 15, 16, 23) have established that rhizosphere bacteria are much more active metabolically and grow faster than bacteria from root-free soil. *Azotobacter*, being a slow-growing organism, cannot compete successfully with these organisms. Another factor which may be operative is the reaction (pH) in the vicinity of the root. The production of CO_2 by both roots and rhizosphere microflora and the elaboration and/or synthesis of acidic substances by roots and microflora could result in a reaction (6.0 or less) which is inhibitory to this rather fastidious bacterium (6, 17). Studies in progress (4) in this laboratory show that when glucose is present in a medium or root extract in sufficient amounts, enough acid is produced by a pseudomonad from the rhizosphere to inhibit *Arthrobacter globiformis*, a typical soil form. The most recent work of Mulder and Van Veen (12) shows that low pH may inhibit the development of rhizobia in the rhizosphere of leguminous plants. Consequently it would appear that an array of conditions unfavorable for *Azotobacter* exist in both soil and rhizosphere, any one or number of which can effectively suppress its development. Most likely the cumulative effects of antibiotic production and competition are responsible. These effects are accentuated in the root zone because of the intense development and activity of its microbial population.

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THE PYRUVIC DEHYDROGENASE SYSTEM OF CLOSTRIDIUM PASTEURIANUM[1]

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Abstract

The pyruvic dehydrogenase system of *Clostridium pasteurianum* has been shown to catalyze a multistep reaction. Spectrophotometric experiments with aged and dialyzed preparations have implicated flavin, the hydrogenase system, and iron in this reaction. Molybdenum, which is not required for the normal action of the hydrogenase system, is needed in some form for the reduction of one-electron acceptors. The carbon monoxide combining component of this system is not of the hemochromogen type and must be in the reduced state before combining with carbon monoxide. A scheme is presented for the liberation of hydrogen from pyruvate by cell-free extracts in the presence of methyl viologen.

Introduction

It has long been known that molecular hydrogen is a metabolic product of the decomposition of carbohydrate by the clostridia and many other bacteria, but only recently has work been done on the pathways by which hydrogen is evolved. *Clostridium butylicum* (5, 6) and *C. butyricum* (24) degrade pyruvate in the presence of phosphate to acetyl phosphate, carbon dioxide, and hydrogen. This reaction is similar to the phosphoroclastic reaction of *Escherichia coli* (19) which produces acetyl phosphate and formate from pyruvate and phosphate. The *Clostridium* enzyme system, however, does not produce hydrogen and carbon dioxide from formate (5), nor does labelled formate exchange with pyruvate, although carbon dioxide does (23). Shug and Wilson (16) reported that hydrogen evolution from pyruvate is not a single-step reaction, but a multistep, complex reaction involving flavin, metals (iron and molybdenum), and the enzyme hydrogenase. This paper presents further work on the role of these components in this pyruvic dehydrogenase reaction (15, 21).

Experimental

Mass cultures of *Clostridium pasteurianum*, strain W-5, were grown in the manner previously described (22). The organisms were harvested using a refrigerated Sharples centrifuge after either the late logarithmic or the early stationary phase of growth had been reached. The cell paste (3–7 g wet weight per liter of culture medium) was suspended in the ratio of one part paste to two parts cold, hydrogen-saturated, 0.05 *M* phosphate buffer, pH 6.8, containing 0.01% cysteine. Treatment of this suspension for 20 minutes at 2° C in a 10-kc Raytheon sonic oscillator resulted in breakage of nearly all of the cells. This suspension was centrifuged for 15 minutes at 25,000 *g* in

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a refrigerated International centrifuge, yielding a fairly clear, yellowish green supernatant (crude extract). The supernatant was transferred to a Thunberg tube, placed under an atmosphere of 100% hydrogen, and stored at 4° C. All preparations, unless otherwise stated, were stored under similar conditions. Dilutions of preparations suitable for assaying were made in cold, hydrogen-saturated, 0.05 *M* phosphate buffer, pH 6.8, containing 0.01% cysteine and 0.04% serum albumin.

The crude preparation was partially purified by centrifuging at 144,000 *g* for 2 hours in a refrigerated Spinco centrifuge (Model L), and the supernatant (144S120) was heated at 60° C for 10 minutes (12). This fractionation procedure resulted in a 7- to 10-fold purification of the hydrogenase activity and a slight decrease in the ability of the preparation to liberate hydrogen from pyruvate. Since further attempts to increase the purification of the hydrogenase activity either by alcohol precipitation or by ammonium sulphate fractionation resulted in the complete loss of the pyruvic dehydrogenase activity, the crude extract and the partially purified 144S120 preparation were used in these studies of the role of metals and hydrogenase in the evolution of hydrogen from pyruvate.

Hydrogenase was assayed by three methods: (*a*) uptake of molecular hydrogen in the presence of suitable acceptors; (*b*) the evolution of hydrogen from reduced methyl viologen; and (*c*) spectrophotometrically, by the reduction of methyl viologen. The pyruvic dehydrogenase system was measured by determining the rate of evolution of molecular hydrogen from pyruvate.

Attempts were made to resolve the iron- and molybdenum-requiring components of the pyruvic dehydrogenase and the hydrogenase systems by dialysis (4° C for 4 hours) in the presence of chelating agents against either 0.05 *M* acetate buffer, 0.02 *M* sodium bicarbonate, or 0.01 *M* ammonium sulphate. To ensure anaerobiosis, 0.01% cysteine was added and Linde high purity nitrogen was continually flushed over the top of the dialysis jar.

Spectrophotometric studies were made using the differential double-beam spectrophotometer (2) and the Beckman (Model DK-2) ratio recording instrument. The rate of change of optical density at 600 mμ (absorption maxima of reduced methyl viologen) was used to assay for both the pyruvic dehydrogenase system (reduction by pyruvate under an atmosphere of nitrogen gas) and hydrogenase activity (reduction with molecular hydrogen). Specially designed cuvettes (1-cm light path) with Thunberg tops were used in these studies.

Results and Discussion

Cell-free preparations of *Clostridium pasteurianum* rapidly decompose pyruvate in the presence of inorganic phosphate to acetyl phosphate, carbon dioxide, and hydrogen. The role of flavin in the hydrogen metabolism of this organism was suggested by the bleaching of the characteristic yellow-green color (absorption maxima 440–450 mμ) of freshly prepared extracts when they were incubated with hydrogen. Figure 1 illustrates the reduction of the flavin of a cell-free extract by the addition of pyruvate. Such preparations would not reduce added pyridine nucleotides with pyruvate but would, however, reduce them slowly with hydrogen. Since the reduction of flavin

in these experiments was very rapid, flavin, but not the pyridine nucleotides, is involved in the evolution of hydrogen from pyruvate.

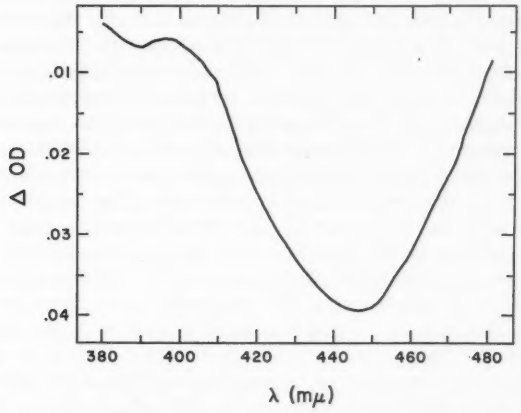


FIG. 1. Difference spectra (steady state oxidized – pyruvate reduced) of crude cell-free extracts in 0.05 *M* phosphate buffer, pH 6.8.

Hydrogenase was shown to be involved in the liberation of hydrogen from pyruvate by use of partially purified cell-free extracts. A partially purified hydrogenase preparation could not liberate hydrogen from pyruvate, while the residue from 50% ammonium sulphate treatment of a crude extract could reduce methyl viologen with pyruvate but not hydrogen, and was not able to evolve hydrogen from pyruvate. The reconstituted system, obtained by combining these two fractions, was able to liberate hydrogen from pyruvate.

Manometric studies revealed that benzyl viologen and methylene blue could serve as electron acceptors in this pyruvic dehydrogenase system and thus inhibit the evolution of hydrogen. When methyl viologen was used in this system a marked increase in the rate and amount of hydrogen evolved resulted, indicating that the dye could substitute in the electron transport system for this reaction (Table I).

TABLE I

Effect of the addition of dyes on the evolution of hydrogen from pyruvate

Preparation	μl H_2 evolved/30 minutes
No additions	40
Methyl viologen: 1 μmole	134
60 μmoles	89
Benzyl viologen: 1 μmole	30
60 μmoles	0
Methylene blue: 1 μmole	25
60 μmoles	0

Cup contents: 150 μmoles phosphate buffer, pH 6.8, 0.1 μmoles cysteine, 100 μmoles pyruvate, 0.1 ml enzyme.

As both iron and molybdenum have been reported as required for the hydrogen-liberating system of clostridia (4, 11, 17), experiments were made in an attempt to learn the locus of function of these metals in this reaction in *C. pasteurianum*. By measuring spectrophotometrically the rate of reduction of methyl viologen with either pyruvate or hydrogen as the source of electrons, evidence was obtained (Fig. 2) that iron as ferrous sulphate stimulates the reduction of methyl viologen with either hydrogen or pyruvate, while molybdenum as silicomolybdate stimulates the reduction of the dye with hydrogen, but not with pyruvate. Silicomolybdate also stimulates the reduction with hydrogen of the one-electron acceptor cytochrome *c* (15). Glenn and Crane (3) showed that in the reduction of cytochrome *c* by xanthine or aldehyde oxidase (9) it was the reduction of the silicomolybdate that was enzyme-catalyzed, the reduction of cytochrome *c* being autocatalytic. As spectrophotometric tests demonstrated that extracts of *C. pasteurianum* will reduce silicomolybdate, it is possible that the reduction of the dyes in these experiments is also autocatalytic. These results, together with the observations of Peck and Gest (12), support the view that molybdenum in some form is required for the interaction of the hydrogenase of clostridia with one-electron acceptors. Neither cobalt nor manganese would substitute for iron or molybdenum in these reactions.

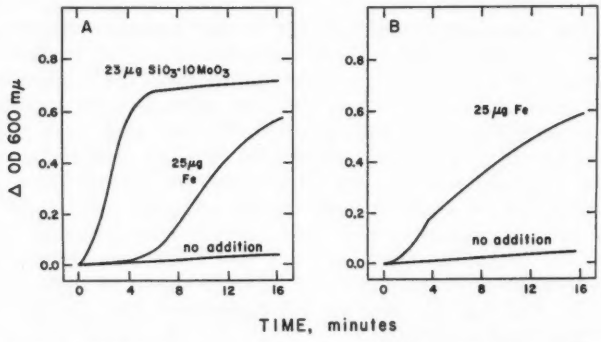


FIG. 2. Metal stimulation of the reduction of methyl viologen by hydrogen and pyruvate.
A. Reaction mixture: 150 μmoles phosphate, pH 7.0, 1 μmole methyl viologen, 0.1 ml enzyme (crude preparation), water to 3.0 ml, atmosphere 100% hydrogen.
B. Reaction mixture: 150 μmoles phosphate, pH 6.8, 1 μmole methyl viologen, 100 μmoles pyruvate, 0.1 ml enzyme, water to 3.0 ml, atmosphere 100% nitrogen.

The age of the preparations was a critical factor in demonstrating these metal stimulations since the pyruvic dehydrogenase system in the extracts deteriorated rapidly even when stored in a concentrated state under hydrogen in the cold. The hydrogenase activity of these extracts, however, was relatively stable over long periods. Extracts approximately 48 hours after preparation showed the described metal stimulations.

Data were obtained with aged and dialyzed preparations which involved iron in the hydrogenase system of this organism. The data in Fig. 3 show an

iron stimulation of the reduction of the two-electron acceptor methylene blue and of the reduction of the one-electron acceptor benzyl viologen. It is to be noted that cysteine has no effect on the rate of reduction of methylene blue but that it does increase the rate of reduction of benzyl viologen. This observation, together with that of Peck and Gest (personal communication) that the addition of 1 mg of neutralized sodium hydrosulphite to the benzyl viologen assay system increases the rate of reduction such that the specific activity approaches that for the methylene blue system, suggests that either a lower potential is required for the reduction of this dye than for methylene blue or, as was suggested by Peck and Gest (11), the oxidized form of this dye is toxic to the enzyme.

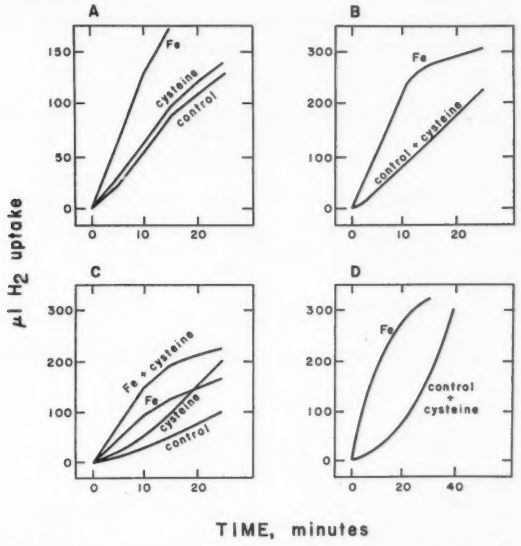


FIG. 3. Effect of additions of iron and cysteine on the methylene blue and benzyl viologen assay systems.

A. Aged 144S120 preparation: 150 µmoles phosphate, pH 6.8, 20 µmoles methylene blue, 10 µmoles Fe^{++}, 0.1 µmoles cysteine, 0.02 ml enzyme.

B. Versene dialyzed preparation: other additions as in A, 0.2 ml enzyme.

C. Aged 144S120 preparation: 150 µmoles phosphate, pH 7.0, 80 µmoles benzyl viologen, 0.1 µmoles cysteine, 10 µmoles Fe^{++}, 0.03 ml enzyme.

D. Versene dialyzed preparation: other additions as in C, 0.3 ml enzyme.

The data in Table II show that the oxygen inactivation of the hydrogenase system (methylene blue assay) is reversible by the addition of reducing agents or by molecular hydrogen provided that the preparation has been exposed to oxygen for only a short period of time. Similar results were obtained using benzyl or methyl viologen as hydrogen acceptors. In an attempt to determine the nature of this oxygen inactivation of the hydrogenase activity, a series of spectrophotometric studies were undertaken. The spectra of preparations under hydrogen or oxygen were compared. The treatment of hydrogen-

reduced extracts with oxygen resulted in the formation of absorption peaks at approximately 445 mμ and 380 mμ, which indicates the presence of oxidized flavins (Fig. 4). Prolonged contact with oxygen of either the diluted crude preparations or the 144S120 preparation resulted in the formation of a precipitate whereas the concentrated crude preparations were self-reducing. Treatment of the former preparations with hydrogen or reducing agents failed to reverse the effect of the oxygen treatment. The inactivation of hydrogenase by brief exposures to oxygen could be reversed by incubation with hydrogen. Such preparations showed that the spectra of the original base line and hydrogenase activity were restored. This spectral demonstration of the reversible nature of the oxidation of flavin suggests its involvement in the oxygen inhibition of hydrogenase. The difference in behavior of the crude and 144S120 preparations towards oxygen may arise from the removal of particulate matter from the 144S120 preparation.

TABLE II

Effect of oxygenation on the inhibition of hydrogenase activity (methylene blue as hydrogen acceptor)

Preparation	Time of oxygen treatment	% inhibition		
		No addition	$+H_2$	+Hydrosulphite
Crude	20 seconds	25	0	0
	5 minutes	100	100	100

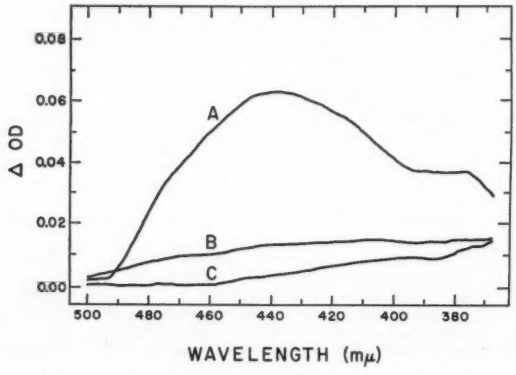


FIG. 4. Difference spectra (oxidized–reduced) showing effect of oxygen on crude cell-free extract.
A. H_2-reduced extract exposed to O_2 for 30 seconds.
B. H_2 bubbled through O_2-treated cuvette.
C. H_2–H_2 control.

Since the results obtained indicate that the evolution of hydrogen is a multistep reaction, a series of inhibitor studies were made to resolve the reaction sequence. The results in Table III lead to the following conclusions:

TABLE III

Effect of inhibitors on the hydrogenase and the pyruvic dehydrogenase system of cell-free extracts of *Clostridium pasteurianum*

Inhibitor	% inhibition					
	Hydrogenase				Pyruvic dehydrogenase	
	Methylene blue	Benzyl viologen	Methyl viologen	Evolution from reduced methyl viologen	Pyruvate reduction of methyl viologen	Evolution of hydrogen from pyruvate
Antimycin A:						
15 μg	0	—	0	0	100	100
Para-chloromercuribenzoate:						
1×10^{-4} *M*	100	100	100	—	—	100
Cyanide:						
1×10^{-3} *M*	24	18	22	—	100	100
3×10^{-2} *M*	79	79	—	72	—	100
Carbon monoxide:						
20%	79	95	—	100	—	90
50%	—	—	100	—	100	—

(*a*) the site of Antimycin A inhibition is prior to the action of the enzyme hydrogenase; (*b*) the inhibition by *para*-chloromercuribenzoate shows that sulphydryl groups are necessary for the activity of the pyruvic dehydrogenase enzyme system; and (*c*) the cyanide and carbon monoxide inhibition implicates heavy metals in both halves of the pyruvic dehydrogenase system with the possibility of a valence change in the heavy metal during the reduction of methyl viologen with pyruvate as the source of electrons. Carbon monoxide inhibition was not reversed by the presence of two reflector lamps (1000 w total) within 14 cm of the bottom of the reaction flasks. Difference spectra ($CO–H_2$) of crude extracts and reduced minus oxidized spectra of alkaline–pyridine-treated preparations (flavin removed by trichloroacetic acid treatment) revealed an absorption maximum at about 415 mμ, but no appreciable absorption in the visible portion of the spectrum (Figs. 5 and 6). The data

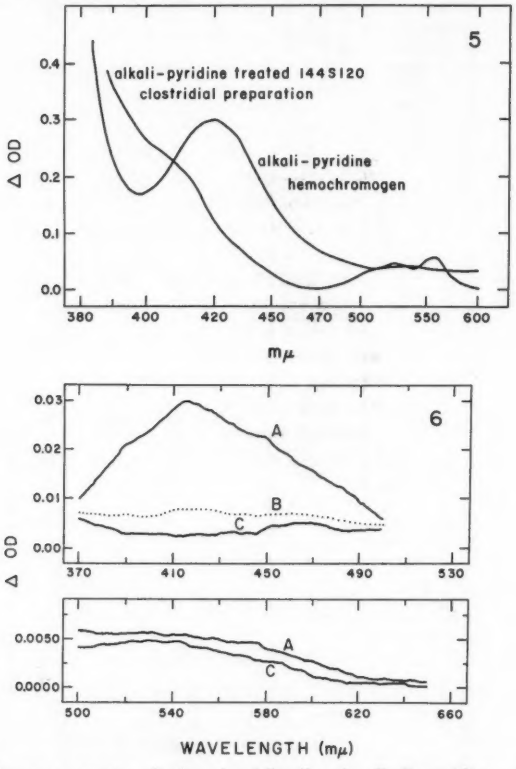


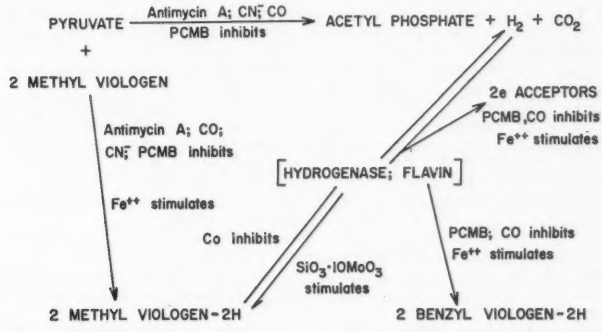
FIG. 5. Difference spectra (reduced–oxidized) of alkali-pyridine derivatives of 144S120 preparation of *Clostridium pasteurianum* and hematin.

FIG. 6. Difference spectra ($CO–H_2$) of whole cells in 0.05 *M* phosphate buffer, pH 6.8.
A. CO bubbled through one of the cuvettes of curve C.
B. H_2 bubbled through CO-treated cuvette.
C. $H_2–H_2$ control.

indicate that the carbon monoxide combining component in *C. pasteurianum* as in *C. butyricum* is not of the hemochromogen type (20). The absorption maximum at 415 mμ was not obtained on treatment of the oxidized preparations with carbon monoxide. This indicates that the component must be in the reduced state before combination with carbon monoxide. Reversal of the reaction between carbon monoxide and the reduced component was obtained by bubbling hydrogen through the cuvette or by replacing the atmosphere with hydrogen.

Conclusions

The pyruvic dehydrogenase system of *Clostridium pasteurianum* has been shown to catalyze a multistep complex reaction. Spectrophotometric experiments with aged and dialyzed preparations have implicated flavin, sulphydryl groups, the hydrogenase system, and iron in this reaction. Evidence was obtained that molybdenum, while not required for the normal action of the hydrogenase system, is needed in some form for the reduction of the one-electron acceptor, methyl viologen (17, 18). The data support the following scheme for the liberation of hydrogen from pyruvate by cell-free extracts of this organism in the presence of methyl viologen:



The implication of iron in the hydrogenase system of *C. pasteurianum* equates this hydrogenase with that of other organisms (7, 8, 12, 13, 14). The role of molybdenum in the hydrogenase system is uncertain (10); however, recent evidence again implicates this metal in nitrogen fixation (1).

Final proof of the role of iron, molybdenum, and flavin in the various reactions of the hydrogenase system will have to await purification of the enzymes involved.

Acknowledgment

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THE ROLE OF PHLORIDZIN IN THE HOST-PARASITE PHYSIOLOGY OF THE APPLE SCAB DISEASE[1,2]

E. H. BARNES[3] AND E. B. WILLIAMS[4]

Abstract

A stimulating substance in apple leaves which enhances the growth of *Venturia inaequalis* (Cke.) Wint. was identified as phloridzin. The metabolism of this compound to phloretin, phloroglucinol, *p*-hydroxyphenylpropionic acid, *p*-hydroxybenzoic acid, and protocatechuic acid is hypothesized on the presence of phloretin and phloroglucinol in the culture filtrates of *V. inaequalis* when it is grown in the presence of phloridzin. The only breakdown product which stimulated the parasite was *p*-hydroxyphenylpropionic acid and it is concluded that its utilization by *V. inaequalis* may be the basis for the stimulatory action of phloridzin.

The effect of these compounds and *m*-inositol on the growth and sporulation of *V. inaequalis* was studied. All of the compounds induced resistant reactions in the 384-1 selection but not in the Geneva variety. This may represent a specificity of the host to the action of these chemicals, indicating that they are correlated with the specificity of the host to races of *V. inaequalis*. Preliminary studies on the effects of these compounds on the metabolism of the host indicated that new phenolic compounds are produced.

Introduction

Previously, Barnes (1) detected a stimulating substance in apple leaves by mpregnating filter-paper disks with various extracts and fractions from healthy leaves and placing them on agar plates seeded with spores of *V. inaequalis*. Crystalline precipitates from water fractions and ethyl acetate fractions were highly stimulating to growth. The phenolic nature and solubility properties of the crystalline precipitates indicated that the material might be phloridzin. This compound and its aglucone, phloretin, may be substrates in the host–parasite interactions since they occur in high concentrations in the host (12, R. R. Goodman, personal communication, and Barnes, unpublished), are specific to the host in their occurrence (12), and are stimulating and inhibitory, respectively, to the growth of the parasite.

Precursors and breakdown products of phloridzin may also be substrates, and therefore additional compounds to be investigated were chosen on the basis of hypothetical schemes for the biosynthesis of the phloridzin molecule (Fig. 1) and its enzymatic degradation (Fig. 2). The scheme for biosynthesis was patterned after the work of Birch *et al.* (3) and Geissman and Hinreiner (9), and enzymatic degradation was hypothesized after the work of Borner (4), Sleeper and Stanier (25), and Stanier *et al.* (27). After initiation of the work,

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Hutchinson *et al.* (11, 12) demonstrated that acetate units and not phloroglucinol are precursors of phloridzin as well as phenylalanine.

The purpose of our investigation was to determine the identity of the stimulating substance and its role in the host–parasite interactions of the apple scab disease.

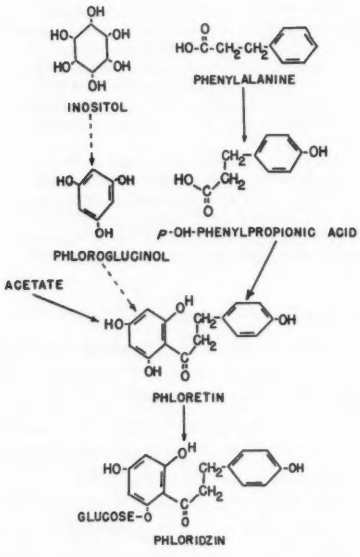

FIG. 1

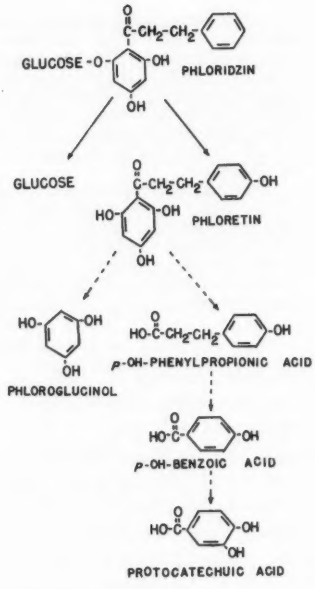

FIG. 2

FIG. 1. A hypothetical scheme for the biosynthesis of phloridzin. The broken arrows indicate a postulated pathway which has been proved to be incorrect whereas the solid arrows indicate an established pathway (11, 12).

FIG. 2. A hypothetical scheme for the metabolism of phloridzin by *V. inaequalis*. The broken arrows indicate a postulated pathway whereas the solid arrows indicate an established pathway.

Materials and Methods

The Host

The host plants used were selected for their value in differentiating genes for pathogenicity in *V. inaequalis* (24, 28). In regard to the three races described (24, 29), selection 384-1 (Mc Intosh × 45-39 (R12740-7A × Delicious)) is susceptible only to race 2 and the Geneva variety (*Malus pumila* var. *niedzwetzkyana* o.p.) is susceptible only to race 3. Reaction classes were those used in previous studies (23, 24) as follows: 1 = few to numerous minute pits without sporulation; 2 = irregular to regular necrotic or chlorotic lesions without sporulation; 4 = extensive lesions with abundant sporulation. Classes 1 and 2 are resistant-type reactions and class 4 is the susceptible-type. Host

material consisted of potted young trees in the greenhouse and young trees in the orchard.

The Pathogen

Isolates representing the three races of *V. inaequalis* were chosen on the basis of pathogenic (24, 28, 29) and growth characteristics. Inoculum was produced by the method used in earlier studies (23) except the wick bottles were seeded with spore suspensions.

Techniques

Inoculations were made by spraying the upper surface of terminal leaves (by means of a No. 15 De Vilbiss atomizer with a pressure of 15 lb/sq. in.) with a conidial concentration of approximately 400,000 conidia/ml. Plants were then placed in a plastic-covered, moist chamber for approximately 40 hours.

Infusion of chemicals was achieved by a modification of the Roach method (22). The third and fourth expanded leaves were trimmed so that 1/8 in. of blade tissue remained on either side of the midrib. These were then immersed in the infusants in vials for periods of 20–24 hours, during which 6–8 ml of infusant was absorbed. Immediately thereafter the plants were inoculated.

Growth responses to chemicals *in vitro* were measured by determining the dry weights of mycelial mats after 24 hours of drying at 80° C. The mats were cultured in 10 ml of media in acid-washed, 25-ml flasks seeded with spore suspensions and harvested at 14 days. All materials were sterilized by autoclaving. In growth and sporulation studies, either a mineral solution and 3% glucose or a mineral solution and 2% Difco malt were employed. The concentrations of minerals were those of Fothergill and Ashcroft (8): K_2HPO_4, 0.004 *M*; $MgSO_4.7H_2O$, 0.002 *M*; NH_4NO_3, 0.375 *M*.

Sporulation responses were determined by measuring conidial concentrations with a hemocytometer. The fungus was cultured in acid-washed test tubes containing 3 ml of a mineral solution and 2% Difco malt with a perpendicular filter-paper wick (2.5×6.5 cm) closely appressed to the side. The concentration of the nutrient medium was adjusted so that the addition of 1 ml of spore suspension to 2 ml of medium diluted it to the proper concentration and brought the total volume to 3 ml. The tubes were slanted for 3 days to permit the conidia to settle and germinate on the wicks and were then placed upright. After 14 days the medium was decanted and 7–8 ml of 1% formaldehyde was added. The tubes were shaken to dislodge the conidia and the spore suspension was filtered through cheesecloth and adjusted to 10-ml volume.

Degradation of compounds by *V. inaequalis* was studied by using appropriate concentrations of the compound in question in 20 ml of mineral–glucose medium in acid-washed 125-ml flasks. After 14 to 30 days' incubation, culture filtrates were collected for chromatographic analysis.

Whatman No. 1 filter paper irrigated with *n*-butanol – acetic acid – water (4:1:1) or benzene – acetic acid – water (organic layer of a 6:7:3 mixture) was used in descending chromatography. The following detection reagents were used (2, 19): bromcresol green, diazotized sulphanilic acid, ferric chloride, and potassium permanganate.

Results

Isolation and Identification of a Substance Stimulating to Fungal Growth

Fifty per cent methanol extracts from the leaves of Geneva and 384-1 were reduced to near dryness under reduced pressure, dissolved in ethyl acetate, filtered, and crystallized by evaporation. The impure crystals were purified by repeated solution in acetone and precipitation with chloroform (21). Melting points were determined for the compound from Geneva and 384-1, commercial phloridzin, and mixtures of these. All sources and mixtures melted between 103 and 112° C, hardened, and melted a second time between 150 and 154° C. Hodgman (10) and Richter (20) report these values to be 106–112° C and 158–160° C. Samples of the stimulating compound from Geneva, 384-1, and commercial phloridzin yielded phloretin, the aglucone, on acid hydrolysis (20). These products were identified by paper chromatography. It is concluded that the stimulating compound is phloridzin.

Metabolism of Phloridzin by V. inaequalis

To elucidate the role of the parasite in the metabolism of the compound, isolates representing the three races of *V. inaequalis* were cultured in the mineral–glucose medium with phloridzin added (2.3×10^{-2} *M*). The culture filtrates were collected after 14 days' incubation, fractionated with ethyl acetate, and chromatographed. Phloretin and phloroglucinol were present in varying concentrations with the highest yields in filtrates of the race 2 culture. A trace of phloretin but no phloroglucinol was present in nonseeded flasks.

The fungus is capable, therefore, of hydrolyzing the phloridzin molecule (Fig. 2) with the release of glucose and phloretin. Further, *p*-hydroxyphenylpropionic acid would be released in equimolar concentrations with the phloroglucinol from the phloretin. Neither this compound nor any other phenolic compound was detected, which indicates that *p*-hydroxyphenylpropionic acid is further metabolized, or the phloretin molecule is cleaved into more than two fragments, or the techniques were unsatisfactory to detect other phenolic compounds.

The Effect of Phloridzin and Related Compounds on the Growth of V. inaequalis

The effects of various compounds on the growth of *V. inaequalis* are presented in Fig. 3. Phloridzin was stimulating; *p*-hydroxyphenylpropionic acid was stimulating at low concentrations and inhibitory at high concentrations; glucose had little effect; phloretin, phloroglucinol, and *p*-hydroxybenzoic acid were inhibitory. The fact that *p*-hydroxyphenylpropionic acid is stimulating at lower concentrations than phloridzin and could not be recovered from culture filtrates indicates that it may be utilized by the parasite and may be directly responsible for the stimulating property of phloridzin. On the other hand, phloridzin itself could be responsible.

Correlation of Physiologic Activity and Pathogenicity

Growth and sporulation were studied with three races of *V. inaequalis* in an attempt to detect race differences in the response of this organism to the precursors and breakdown products of phloridzin. One isolate of each race was tested. When all three responded similarly to a given compound, it was

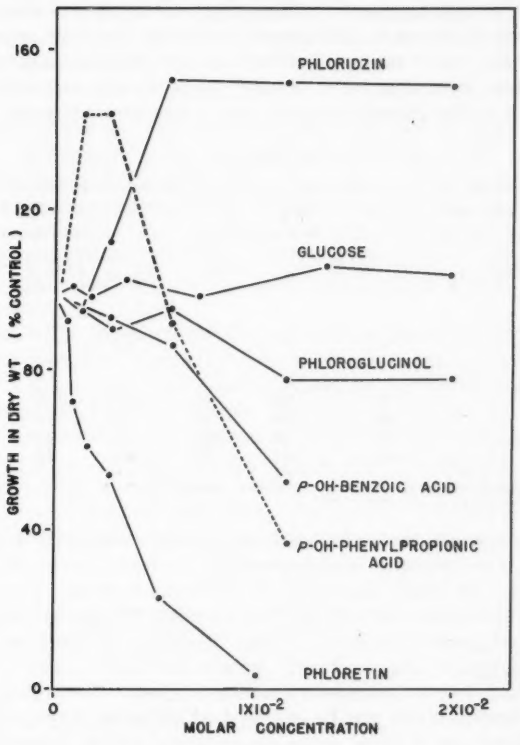

FIG. 3. The response of *V. inaequalis* to phloridzin and its hypothetical breakdown products. The fungus was cultured in malt extract.

TABLE I

Growth responses of ascospore isolates of *V. inaequalis* to certain compounds

Pathogenicity and growth	Concn., $\times 10^{-3}$ *M*	Parent isolates		Ascospore isolates			
		356–2	651	1536–1	1536–3	1536–5	1536–7
Pathogenicity*							
Geneva	—	−	+	+	+	−	−
384–1	—	+	−	+	−	−	+
Growth†							
p-OH-Cinnamic acid	2.50	135	90	79	89	126	81
Phloroglucinol	1.15	80	110	93	122	67	87
Tyrosine (DL)	2.00	80	135	104	77	110	114
Alanine (DL)	15.00	180	65	110	82	112	45

*The symbols + and − indicate virulence and avirulence, respectively.
†The numbers are the average dry weights/flask in mg.

inferred that this response was independent of the genes governing pathogenicity. Where there were differences, cultures from an ascospore set of four biotypes, for which segregation for pathogenicity on Geneva and 384-1 was known, were examined to determine whether the response of the four-biotype isolates to the chemical segregated in the same manner.

TABLE II

Sporulation responses of ascospores isolates to *V. inaequalis* to certain compounds

Pathogenicity and sporulation	Concn., $\times 10^{-3}$ *M*	Parent isolates		Ascospore isolates			
		356–2	651	1536–1	1536–3	1536–5	1536–7
Pathogenicity*							
Geneva	—	−	+	+	+	−	−
384–1	—	+	−	+	−	−	+
Sporulation†							
m-Inositol	5.75	60	96	111	106	89	84
Phloretin	2.90	188	80	55	79	89	45
Phloroglucinol	5.75	65	103	89	39	72	35
Phloridzin	5.75	21	91	99	42	32	104

*The symbols + and − indicate virulence and avirulence, respectively.
†The numbers indicate numbers of spores/mm³.

When the response to a chemical segregates differently from genes for pathogenicity, the response is independent of those genes. However, when the response to a chemical segregates in the same manner as genes for pathogenicity, then that response may be due to either the genes for pathogenicity or closely linked genes. There is also one chance in six that two independent genes could segregate concomitantly within the same ascus since there are six ways in which two independent genes may assort themselves.

A 1:1 distribution could not be established for growth responses (Table I). Apparently there are several genes segregating which govern growth, and therefore correlations of growth responses with genes for pathogenicity cannot be achieved by these procedures. In sporulation responses, a 1:1 segregation seemed to occur (Table II). In a few of the data, the level of significance was such that it may or may not be a 1:1 segregation. The effect of *m*-inositol on sporulation was the only difference which correlated with known genes for pathogenicity. The gene which conditions pathogenicity on Geneva segregated like the response to *m*-inositol with the one ascospore set examined. The sporulation of isolates with the allel for avirulence on Geneva was inhibited whereas it was not with isolates with the allel for virulence. Since these studies, further tests with the other ascus sets have demonstrated that the response to *m*-inositol does not always segregate with the genes for pathogenicity (D. MacClennen, Purdue University, personal communication). The response is due to either loosely linked genes or independent genes which fortuitously segregated in a like manner.

Although this particular attempt to correlate a physiologic response with a pathologic response has failed, it is worth while to propose additional techniques necessary to make such a correlation. Once the segregation of genes within a few asci showed a continued correlation the next step would be to

irradiate conidia and screen for pathogenic mutations, followed by a test to determine whether the change in pathogenicity occurred concomitantly with a change in physiologic response. The final step would be to perform the necessary genetic test crosses to determine whether only one gene had mutated.

The Effect of Phloridzin and Related Compounds on Host-reaction to the Parasite

Geneva and 384-1 were infused prior to inoculation with phloroglucinol, *m*-inositol, *p*-hydroxyphenylpropionic acid, and *p*-hydroxybenzoic acid. Different concentrations were infused to determine the highest concentrations (those listed in Table III) that could be used without inducing signs of injury. The phloridzin and phloretin were not infused since they occur in high concentrations in apple leaves (2–2.5% of the dry weight) and are relatively insoluble in water.

The infusion with these materials induced resistant reactions in 384-1 inoculated with race 2 (Table III). Many resistant reactions were on half-leaves while the other half of the leaves exhibited susceptible reactions. This may have been a reflection of the distribution of the materials infused. Most of the resistant reactions were on leaves approaching the mature-resistance stage, indicating that a metabolic balance is most easily disrupted during this stage of development. This is similar to the observation described by Kirkham (14) that the maximum effect of phenolic compounds on the size and number of lesions was on leaves approaching this stage of development.

TABLE III

The effects of infused compounds on the disease reactions of Geneva and 384–1 apple varieties to *V. inaequalis*

Infusant	Concn., $\times 10^{-2}$ *M*	Reaction of Geneva*		Reaction of 384–1*	
		Race 1	Race 3	Race 1	Race 2
Water	—	2	4	1	4
m-Inositol	6	2	4	1	1
Phloroglucinol	3	2	4	1	1
p-OH-phenylpropionic acid	2	2	4	1	1
p-OH-benzoic acid	2	2	4	1	1

*Numbers indicate the average reaction class of 6 and 9 replications of the Geneva and 384-1 varieties, respectively. In most cases, leaves exhibiting type 1 reactions also exhibited four reactions on opposite halves of the leaves.

None of the chemicals induced resistant reactions in Geneva to race 3 in two replicated experiments (Table III). This could indicate a failure to infuse sufficient quantities of the materials or a real difference between this variety and 384-1. Geneva and 384-1 were infused and inoculated with race 1, which induces resistant reactions on both varieties. No alteration in reaction types was observed with either variety (Table III).

A chromatographic examination of tissues of 384-1 infused with the various compounds indicated that the compounds induce changes in the metabolism of the host. Three additional phenolic compounds were detected with *n*-butanol – acetic acid – water as the solvent. One, at an R_F of 0.23, was present only in tissues infused with phloroglucinol. A second one which was found in tissues infused with *m*-inositol, at an R_F of 0.65, appeared to be

identical with one, found at an R_F of 0.62, in tissues infused with *p*-hydroxyphenylpropionic acid. In tissues infused with the latter compound, there was a prominent spot, at an R_F of 0.87, which was *p*-hydroxyphenylpropionic acid. A compound with a similar R_F and reactivity with reagents was present in tissues infused with *m*-inositol. This indicates that perhaps *m*-inositol enters the pathway of synthesis of *p*-hydroxyphenylpropionic acid or affects the metabolism of the host so that this compound is produced.

Discussion

Kirkham (14) found that injections of the water-soluble, aromatic constituents of apple were inhibitory to the size and number of apple scab infections but were not inhibitory when injected with urea. Injections of urea alone stimulated development. On the basis of these results, Kirkham and Flood (17) and Flood (7) examined the inhibitory properties of analogues of host constituents *in vitro* and found that *o*-hydroxycinnamic acid and cinnamic acid were inhibitory to sporulation. They concluded that the —CH=CH—COOH group appeared to be necessary for inhibition of sporulation but that the effect of this group was modified by the number and position of hydroxyl groups on the adjacent benzene ring. Kirkham (15, 16) then tested the effects of normal phenolic host metabolites on sporulation *in vitro* and the development of the disease *in vivo*. Although there were no differences between resistant and susceptible varieties in the major phenolic components, these compounds inhibited the parasite when injected into the same varieties from which they were extracted. The *in vitro* inhibition with these phenolic extracts, however, was independent of the relative susceptibility or resistance of the varieties.

The findings of Kirkham and Flood cannot be directly compared with ours because they measured changes in the number and size of lesions whereas we measured changes from one reaction class to another. The existence of a mechanism controlling numbers of lesions which is independent of the mechanism(s) conditioning kinds of lesions has been suggested by Barnes (1). He found that the percentages of germination and appressorial formation of conidia on intact leaves were under the influence of the host but that these affects were independent of the compatibility of the host–parasite combinations.

Phloridzin and its hypothetical precursors and breakdown products may play an important role in the host–parasite interactions of the apple scab disease for the following reasons: (1) phloridzin occurs in high concentrations in the host; (2) phloridzin is specific to the host in its occurrence; (3) the parasite is stimulated by phloridzin; (4) the parasite can metabolize phloridzin; and (5) hypothetical precursors and breakdown products of phloridzin are effective in the induction of resistance.

If the failure of phloridzin and related compounds to induce resistance in Geneva is a real difference between this variety and 384-1, then the above hypothesis is further supported since a host specificity in response to chemicals and parasites would indicate correlation. Some of the compounds themselves might not be directly related to any of the natural mechanisms of the host–parasite interactions, but it is clear that they induce biochemical changes in

the host tissues which parallel changes in the susceptibility of the host tissues. Perhaps some of these induced biochemical changes are directly related to the host–parasite interactions. The action of these chemicals in the induction of resistance, then, can be interpreted to be their relationship to, or effect on, the synthesis and/or degradation of phloridzin.

Our investigations and others do not preclude alternative hypotheses. The information indicates a relationship of resistance to lignin as adequately as it does to phloridzin. All of the compounds considered, except phloroglucinol, which induce resistance are related to lignin synthesis as either structural units and breakdown products, or analogues thereof. Phenylalanine (5, 6), cinnamic acid (6), *p*-hydroxycinnamic acid (18, 26), *p*-hydroxybenzoic acid (6, 26), and *m*-inositol (13) have been reported as lignin precursors. The action of these compounds in the induction of resistance may be their relationship to, or effect on, the synthesis and/or degradation of lignin.

Disease reactions are a result of the differences in the susceptibility of varieties of the host and in the virulence of isolates of the parasite. To believe that more than one pathway is involved is not unreasonable and both phloridzin and lignin could be related to the host–parasite interactions by virtue of the intermediate compounds in the pathway of their synthesis and degradation.

Acknowledgments

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FACTORS INFLUENCING INACTIVATION OF INFECTIVITY AND HEMAGGLUTININ OF INFLUENZA VIRUS BY GAMMA RADIATION[1]

JOHN R. POLLEY

Abstract

Factors influencing the effects of gamma irradiation on influenza A(PR8) virus suspensions have been investigated. In purified virus suspensions in physiological saline, the hemagglutinin was destroyed more rapidly than the infectivity. The addition of reagents such as histidine, sodium *p*-aminohippurate, ascorbic acid, or cystine to the saline suspension reversed this effect. It was also found that the effect of a given amount of gamma radiation on the infectivity and hemagglutinin was similar regardless of whether the radiation was administered as a single dose or as two or four divided doses on different days. It is possible to calculate the amount of radiation required to destroy the infectivity and yet retain most of the hemagglutinin content. If a given dose of radiation has been insufficient to produce complete virus inactivation, the suspension can be subjected to a further dose, the amount of which can be exactly calculated, without destroying the hemagglutinin. These experiments were repeated with other strains of influenza A and B with similar results. The application of this work to the preparation of virus vaccines is being investigated and will be reported later.

Introduction

Gamma irradiation can be used to inactivate viruses and, under appropriate conditions, antigenicity can be retained (1, 2, 3). For example, Jordan and Kempe (1) performed the irradiation with the samples packed in dry ice; virus suspensions, partially purified by differential centrifugation and resuspension in buffers, were found to be more readily inactivated than unpurified suspensions. On the other hand, with virus present in tissue extracts the partial purification by centrifugation had little effect (3). Kaplan (2) irradiated vaccinia virus suspensions but found that "the immunogenicity of our inactivated preparations was not closely related to the inactivating dose of γ-rays"; in fact, a given dose of radiation did not always produce the same result.

It was shown in this Laboratory that, with liquid samples, gamma irradiation could be used to inactivate consistently the virus present in soluble diagnostic antigens, thus giving a safe product for laboratory use (3). The preparation of viral antigens and vaccines, through inactivation by chemical agents, is seldom easy because of the difficulty of controlling the inactivation process precisely enough to retain the antigenicity while destroying the infectivity. From this work on the gamma irradiation of diagnostic antigens, one fact of possible importance to virus vaccine preparation was found, namely, that it was possible to apply an additional calculated amount of radiation to destroy residual infectivity without causing loss of antigenicity. Because of this greater ease of controlling the virus inactivation brought

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Contribution from the Virus Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa.

about by gamma irradiation, a study has been made of factors influencing the effect of gamma irradiation on influenza virus suspensions with a view to developing a procedure for the preparation of vaccines.

Materials and Methods

The irradiation of the virus suspensions was carried out at the Commercial Products Division of Atomic Energy of Canada, Limited, Ottawa, using a commercially available cobalt-60 cell. For irradiation, 10-ml samples of these suspensions were placed in small glass vials with bakelite screw caps. The vials were placed in a carrier in the gamma cell. The gamma cell used in these experiments emitted radiation at a dosage of 1.25×10^6 rads/hour. Since the rate of emission is essentially constant, the dosage administered is proportional to the time of exposure. Vials were removed after various periods of exposure and were stored overnight at 4° C. The irradiated virus suspensions were then tested for hemagglutination titer and infectivity, as described before (4).

Virus suspensions were prepared by centrifuging the freshly harvested allantoic fluid containing influenza A(PR8) virus at 10,000 r.p.m. (8700 *g*) for 1 hour in the No. 30 rotor of a Spinco model L centrifuge. The sediment was resuspended in a quantity of physiological saline so as to produce a hemagglutination titer of about 1:2560 per ml.

Results

Preliminary experiments indicated that with these partially purified virus suspensions, the hemagglutinin was being destroyed more rapidly than the infectivity; this was more pronounced in some experiments than in others. The possibility of adding to the virus suspension in saline some reagent which would give a higher degree of protection to the hemagglutinin than to the infectivity during gamma irradiation was investigated. The effect of three chemically different reagents, added to samples of the virus suspension, on the rate of destruction of the hemagglutination titer and the infectivity was studied quantitatively. A carbohydrate (sucrose), a straight-chain amino acid containing the guanidine group (arginine), and a heterocyclic amino acid (histidine) were added singly at a concentration of 0.5% in separate samples of an influenza A virus suspension prepared as above. These samples were irradiated with various doses and then tested as before. The effect of the added reagents on the rate of destruction of the infectivity by gamma irradiation is shown in Fig. 1 and on the rate of destruction of the influenza A hemagglutinin in Fig. 2.

It can be seen that the presence of arginine or histidine in the suspending medium has decreased the rate of virus inactivation by gamma irradiation; sucrose has had little effect. Similarly, arginine and histidine have increased greatly the stability of the influenza A virus hemagglutinin to gamma irradiation, while sucrose has had less effect. The ratio of the radiation dose producing complete destruction of the infectivity to the dose producing complete elimination of the hemagglutinin was calculated for each of these reagents, with the following results: for physiological saline, 5.3; for sucrose, 3.0; for arginine, 1.6; and for histidine, 0.2. Thus, only the latter compound has been found

effective in providing selectively greater protection to the hemagglutinin during inactivation by gamma irradiation.

An additional number of reagents were then studied under similar conditions as described above. All these reagents were added to samples of saline virus suspension to give a concentration of 0.2% in each. The results are shown in Table I. It can be seen from this table that carbohydrates, such as glucose,

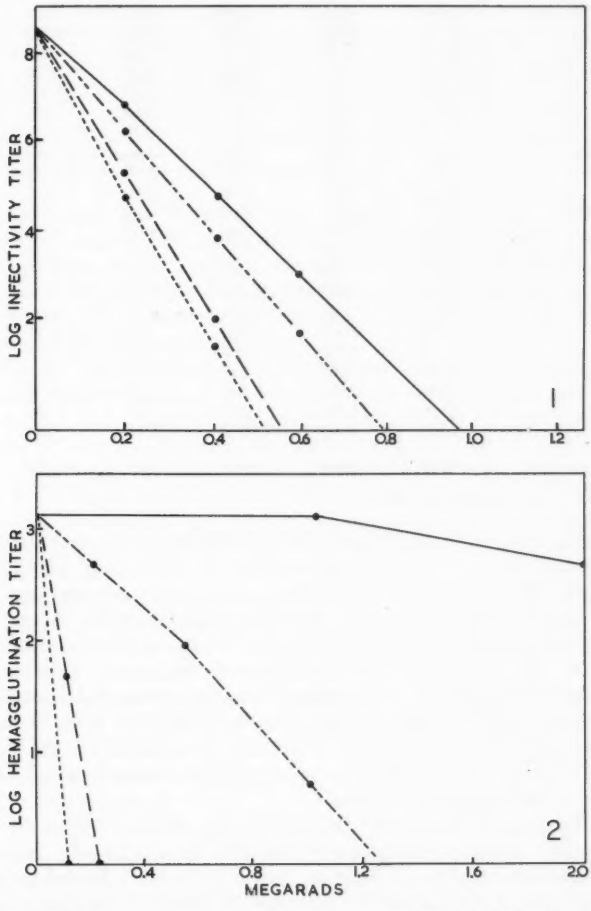

FIG. 1. Effect of various reagents, added to the saline suspension of influenza virus, on the rate of inactivation by gamma irradiation.

FIG. 2. Effect of various reagents, added to the saline virus suspension of influenza virus, on the rate of destruction of the hemagglutinin by gamma irradiation.

TABLE I

Capacity of various reagents, added to influenza A virus suspension, to protect the hemagglutinin during gamma irradiation

Reagent added (0.2% concn.)	With radiation dose (rads) of:					
	0	0.5×10^6	1.0×10^6	2.0×10^6	3.0×10^6	4.5×10^6
O (control)	2560*	<20	<20	<20	<20	<20
Histidine	2560	2560	2560	2560	1280	160
Cystine	2560	2560	2560	640	160	<20
Tyrosine	5120	2560	2560	320	<20	<20
Methionine	5120	5120	2560	2560	1280	320
Aniline	2560	2560	2560	640	<20	<20
Tryptophan	2560	2560	1280	320	<20	<20
Sulphanilamide	5120	2560	1280	640	80	<20
Phenylalanine	2560	1280	640	<20	<20	<20
Calf serum	5120	2560	320	<20	<20	<20
Bovine albumin	2560	2560	320	<20	<20	<20
Peptone	2560	1280	160	<20	<20	<20
Gelatin	2560	160	<20	<20	<20	<20
Glucose	2560	160	<20	<20	<20	<20
Sucrose	2560	160	<20	<20	<20	<20
Inulin	2560	80	<20	<20	<20	<20
Ascorbic acid	2560	2560	1280	320	<20	<20
Pyridine	2560	1280	1280	160	<20	<20
Proline	2560	1280	160	<20	<20	<20
Uracil	2560	2560	160	<20	<20	<20
Allantoin	5120	2560	1280	640	80	<20
Cysteine	2560	2560	2560	2560	320	160
Sodium *p*-aminohippurate	2560	2560	2560	640	320	40

*Expressed as hemagglutinin units per ml.

sucrose, and inulin, have had little effect. Proteins, such as calf serum and bovine albumin, afford only a small measure of protection. Simple straight-chain amino acids, not containing sulphur, such as glycine and arginine, have a relatively small effect. The most effective compounds for the protection of the hemagglutinin during gamma irradiation are the sulphur-containing amino acids (cystine, cysteine, and methionine) and compounds containing a ring structure such as histidine, tryptophan, tyrosine, phenylalanine, sodium *p*-aminohippurate (PAH), and sulphanilamide. Ascorbic acid, a well-known antioxidant, also exerts a protective action. Irradiation of the suspensions containing methionine or cysteine produced unpleasant odors, suggesting the rupture of carbon–sulphur bonds.

The relationship of the concentration of the reagent added to the saline virus suspension to the protective effect on the hemagglutinin during gamma irradiation was investigated. Histidine and PAH were selected as two of the most promising compounds for this purpose (Table I). Virus suspensions were prepared containing different concentrations of these reagents. Samples of each suspension were then irradiated with various doses and tested as before. The results are shown in Table II.

It can be seen from Table II that increasing the concentration of histidine or PAH in the virus suspension has given increased protection to both the

TABLE II

Effect of gamma irradiation on the hemagglutinin and infectivity of influenza A virus suspensions containing various concentrations of histidine or sodium *p*-aminohippurate (PAH)

	With histidine concentration of:			With PAH concentration of:		
Radiation (rads)	0.05%	0.2%	0.8%	0.05%	0.2%	0.8%
0	5120*	5120	5120	5120	5120	10240
0.25×10^6	5120	5120	5120	5120	5120	10240
0.5×10^6	5120†	5120	5120	5120	5120	10240
1.0×10^6	2560†	5120†	5120	2560†	5120†	10240
2.0×10^6	1280†	2560†	5120†	640†	1280†	10240†
4.0×10^6	40†	320†	1280†	40†	160†	1280†
6.0×10^6	<20†	<20†	80†	<20†	<20†	<20†

*Expressed as hemagglutinin units per ml.
†The virus suspension was noninfective at this dose.

hemagglutinin and the infectivity on gamma irradiation. While this was expected it was hoped that there might be a relatively greater protection for the hemagglutinin as the reagent concentration was increased but such was not the case. From the experiments described above it appears that the addition of about 0.2% histidine to the influenza virus suspension gave the optimal protective effect to the hemagglutinin.

The effect of inactivation by divided doses of radiation was also studied. In this experiment samples of a common pool of influenza A virus suspension were irradiated with a given dose of radiation in one of three ways: (*a*) one single dose; (*b*) two half-doses a day apart; and (*c*) four quarter-doses each a day apart. Between periods of irradiation the samples and controls were stored at 4° C. Upon completion of irradiation, the samples were tested for infectivity and hemagglutination titer as before. An influenza virus suspension containing 0.2% histidine was also included in this experiment. The results are shown in Table III.

From these data it is evident that there was no significant difference in the effect produced by administering a given amount of radiation as a single dose

TABLE III

Effect on the hemagglutinin and infectivity titers of a given amount of gamma radiation administered either as a single dose or as two or four divided doses on successive days

Virus suspension	Radiation (rads)	Single dose		Two half-doses		Four quarter-doses	
		HA*	Inf.†	HA	Inf.	HA	Inf.
In saline	0	5120	$10^{-9.5}$	5120	$10^{-9.5}$	5120	$10^{-9.5}$
	100,000	5120	$10^{-5.0}$	2560	$10^{-4.5}$	2560	$10^{-5.0}$
	200,000	1280	$10^{-3.0}$	640	$10^{-2.7}$	1280	$10^{-2.5}$
	400,000	<20	$10^{-0.5}$	40	$10^{-0.5}$	<20	0
In saline +0.2% histidine	200,000	5120	$10^{-7.5}$	5120	$10^{-7.0}$	5120	$10^{-7.5}$
	400,000	5120	$10^{-6.5}$	5120	$10^{-5.2}$	5120	$10^{-5.0}$
	800,000	5120	$10^{-3.5}$	5120	$10^{-3.5}$	5120	$10^{-3.0}$

*Expressed as hemagglutinin units per ml.
†Infectivity titer.

or in divided doses. Apparently the inactivation process stops almost at once upon removal of the virus suspension from exposure to the radiation. Thus, it appears that the inactivation produced by successive exposures to radiation has been additive in these experiments, again indicating the large measure of control that can be achieved by inactivation with gamma radiation.

Discussion

From the preliminary experiments it appeared that the presence of an unknown impurity in the virus suspensions gave some protection to the hemagglutinin during gamma irradiation. The capacity of a number of different reagents, when added to the influenza virus suspension, to protect the hemagglutinin during gamma irradiation is of practical importance in the inactivation of virus suspensions for vaccines. The most effective compounds were the sulphur-containing amino acids and the compounds containing a ring structure. It is interesting to compare the relative protective effects of the ring compounds tested. For example, phenylalanine and tyrosine have a similar structure except that tyrosine has a hydroxyl group on the benzene ring and is more effective than phenylalanine. Similarly, sulphanilamide and *p*-aminohippurate both contain an amino group on the benzene ring and aniline itself is quite effective, showing that the protective action does not depend only, if at all, on the aliphatic side chain. Ascorbic acid, an antioxidant containing neither a sulphur atom nor an aromatic ring, also exhibited a protective action on the hemagglutinin during gamma irradiation, suggesting that an interference with oxidizing conditions in the medium during irradiation had taken place. The ring compounds containing the aniline structure may also exert an antioxidizing action as they show some darkening in color on irradiation with over 1×10^6 rads.

Since most of the reagents added to the virus suspensions are not of greatly different molecular weight, it appears that the explanation for their varying protective effect for the hemagglutinin during irradiation cannot be due to a blocking of the direct action of gamma rays, but is more likely due to an interference with chemical action. The above observations would suggest that it is the formation of a strongly oxidizing medium during irradiation that accounts for much of the destruction of both the hemagglutinin and the infectivity. In a purified virus suspension this oxidizing action destroys the hemagglutinin more rapidly than the infectivity. The addition of certain reagents, such as histidine, PAH, cystine, and ascorbic acid, affords relatively greater protection to the hemagglutinin than to the infectivity. Thus, it is possible by means of gamma radiation to destroy the infectivity of an influenza virus suspension while retaining most of the antigenicity.

Of further practical importance is the fact that the infectivity can be reduced to approximately the same extent regardless of whether the radiation is administered as a single dose or in divided doses. From the kinetics of the virus inactivation it is possible to calculate the amount of radiation required to destroy the infectivity and yet retain most of the hemagglutinin content. If a given dose of radiation has been insufficient to produce complete virus inactivation, the suspension can be subjected to a further dose, the amount of which can be exactly calculated from the inactivation curve.

The PR8 strain of influenza A virus was used in the above experimental work but similar results were obtained with other strains of influenza A and influenza B virus.

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BIOCHEMICAL CHANGES OCCURRING IN A MINIMAL MEDIUM DURING GROWTH AND SPORULATION: COMPARISON OF SPOROGENIC BACILLUS CEREUS WITH ASPOROGENIC MUTANTS[1]

G. BESKID[2] AND D. G. LUNDGREN

Abstract

Chemical changes in a minimal synthetic medium were studied during growth and sporulation of normal and temperature-sensitive mutant cultures of *B. cereus* at 28° C. Similar chemical studies were made when the cultures were grown at 37° C; the mutants were asporogenic (nonsporulating) at this temperature.

Concurrent events, essentially the relationships between spore formation, the consumption of zinc, and consumption of manganese, were noted in sporulating and nonsporulating cultures. Other components of the medium also investigated included: glucose, amino-N, inorganic-P, potassium, iron, and magnesium. Metabolic products detected in the medium included: diaminopimelic acid, dipicolinic acid, ammonia-N, amide-N, peptide-N, and organic-P.

Introduction

The initial report of the investigation of sporulation in *Bacillus cereus* and induced nonsporulating mutants of this organism characterized the culture's growth and sporulation habits at 28° C and 37° C (10, 11). The cultural conditions of this study were such that a single cycle of events occurred (i.e., spore ⟶ vegetative cell ⟶ spore) in the parent organism at both 28° C and 37° C. The synthetic medium employed was a minimal one, allowing a cycle of events eventuating first in germination and vegetative growth, followed by endospore formation. Such a medium was therefore adequate for the isolation of mutants which were no longer able to sporulate because of genetic interference.

The three U.V.-induced mutants when grown in the minimal medium at 37° C were nonsporulating, whereas the parent cells sporulated to a 90–100% level. These nonsporulating cells were called asporogenic. At 28° C, however, it was noted that these same mutant cells grown under identical cultural conditions were capable of sporulation. No observable differences in growth were noted between the three mutants and the wild type cells grown at the two temperatures.

It was hoped that some clues relative to the involvement of specific biochemical events concerned with bacterial sporulation might be uncovered by assaying for chemical changes occurring in the synthetic medium. Chemical changes were investigated during various stages of culture development; i.e., (1) during early growth, (2) at the peak of growth, (3) during early spore formation, and (4) late in spore formation. Hashimoto *et al.* (8), using phase

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contrast microscopy and electron micrography, have also observed successive stages in spore development. In asporogenic cultures, the latter two stages were designated as "abortive" sporulation. The present report describes the chemical changes in the minimal medium when inoculated with sporogenic or asporogenic cells, and has been reported in a preliminary account (2).

Materials and Methods

Organisms and Cultural Procedures

The sporogenic (normal) organism used in the investigation was *B. cereus* (A.T.C.C. No. 4342). The three asporogenic mutants of the parent investigated were strain Nos. 40, 52, and 54. The minimal medium for growth and sporulation contained: glucose, 5 g; glutamic acid, 10 g; glycine, 0.1 g; K_2HPO_4, 0.5 g; KH_2PO_4, 0.5 g; $MgSO_4.7H_2O$, 0.2 g; $FeSO_4.7H_2O$, 0.01 g; $MnSO_4.4H_2O$, 0.01 g; NaCl, 0.01 g; $ZnSO_4.7H_2O$, 0.013 g; $CaHPO_4$, 2 ml (satd. sol.); distilled water, to 1 l.; pH adjusted to 6.7 after autoclaving.

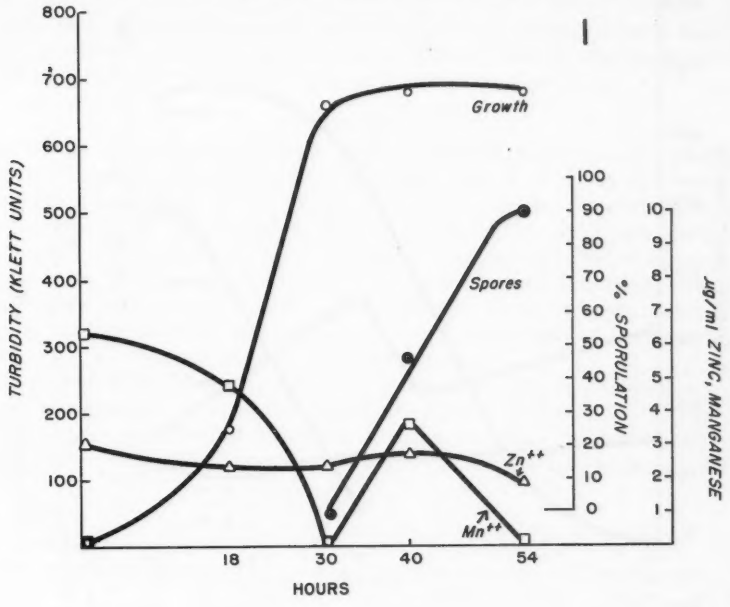
Preparation of inocula and cultural conditions were as previously described (11). Growth and sporulation were studied in shaken cultures using 50 ml of the minimal medium dispensed in 250-ml Erlenmeyer flasks at 28° C and 37° C. Chemical changes occurring at 28° C in the minimal medium during growth (0–30 hours) and sporulation (30–54 hours) of normal and mutant cultures were studied. Identical medium studies were made after incubation of the same cultures at 37° C (growth, 0–18 hours), at which temperature only the normal *B. cereus* cultures sporulated (18–30 hours).

Growth was measured turbidimetrically using a Klett–Summerson photoelectric colorimeter and a blue, No. 42 filter. The percentage of spores was determined by a microscopic examination of heat-fixed films stained with malachite green and safranin. Values reported represent cells having fully formed refractile spores. Cells and/or spores were separated from the medium at different stages of growth and sporulation by centrifugation, using a refrigerated centrifuge. The cell-free medium was collected in sterile flasks and stored in a refrigerator. Aliquots of the medium were periodically removed for the various chemical analyses.

Chemical Assays

Glucose was determined colorimetrically using the dinitrosalicylic acid method (13). Diaminopimelic acid was determined by paper chromatography (3), using ninhydrin as the color developer (17). The colorimetric method of Janssen *et al.* was employed for the assay of dipicolinic acid (9). Total-N, ammonia-N, amide-N, amino-N, and peptide-N were each determined by conversion to NH_3; the NH_3 was then measured colorimetrically by the phenol–hypochlorite method (12). Both inorganic-P and total-P assays were made using the method of Fiske and SubbaRow (5); organic-P was calculated by difference. Other chemical determinations included: the dithizone method for zinc (18), the dipicrylamine method for potassium (1), the *o*-phenanthroline method for iron (18), the permanganate method for manganese (14), and the thiazole yellow method for magnesium (19).

Initial measurements of the various chemical components of the minimal medium were made on the uninoculated medium. All assays were made in



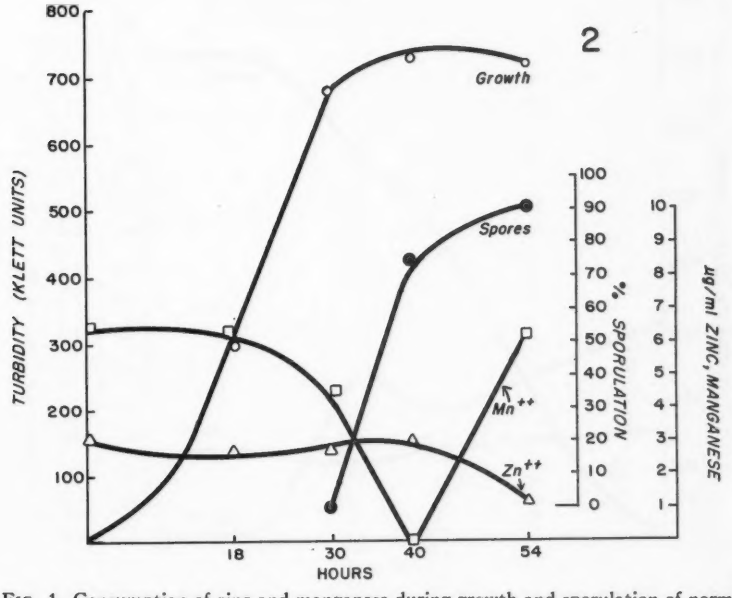


FIG. 1. Consumption of zinc and manganese during growth and sporulation of normal *B. cereus* cells cultured at 28° C.

FIG. 2. Consumption of zinc and manganese during growth and sporulation of *B. cereus* mutant No. 52 cells cultured at 28° C.

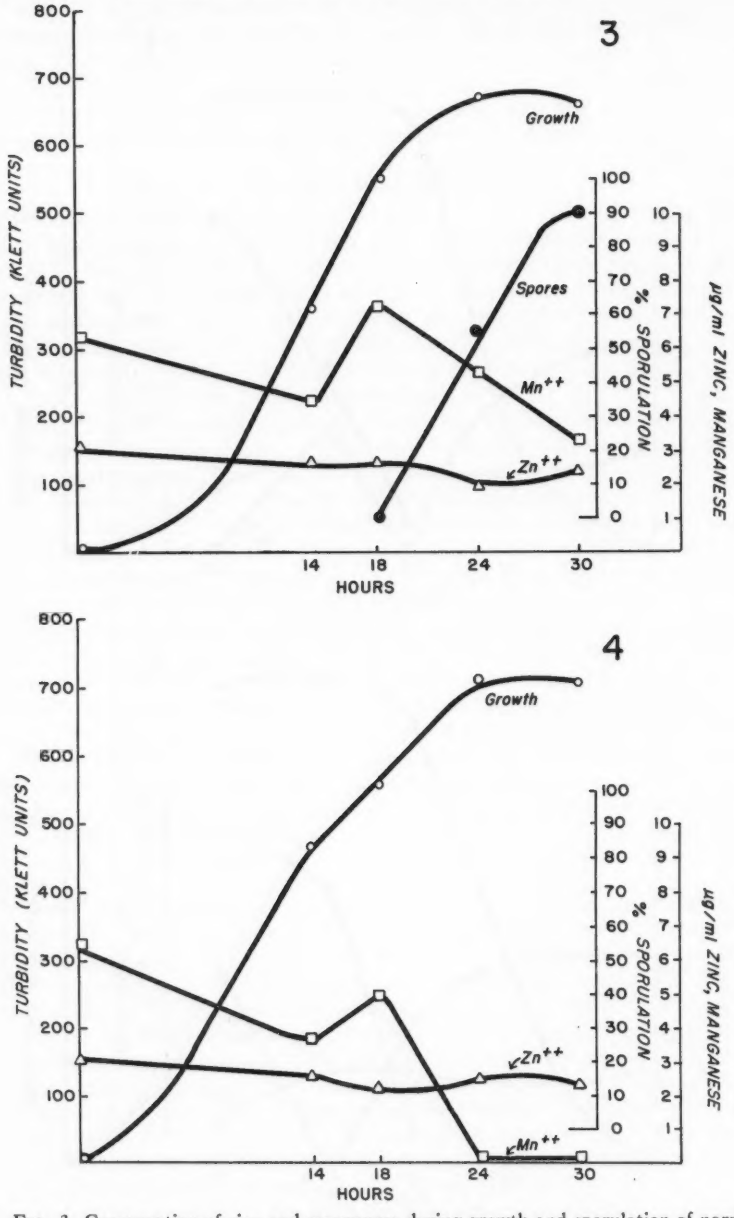


FIG. 3. Consumption of zinc and manganese during growth and sporulation of normal *B. cereus* cells cultured at 37° C.

FIG. 4. Consumption of zinc and manganese during growth and "abortive" sporulation of *B. cereus* mutant No. 52 cells cultured at 37° C.

duplicate and results expressed as the average value. Optical density and per cent transmittance were measured with a Bausch and Lomb Spectronic 20 colorimeter. Per cent recoveries were made for each of the tests.

Results

The results of chemical analyses of media incubated at 28° C and supporting growth and sporulation of normal cells and cells of temperature-sensitive mutant No. 52 are shown in Figs. 1 and 2 and also in Table I. Results of similar tests carried out on inoculated media but incubated at 37° C are shown in Figs. 3 and 4 and in Table II. At 37° C, growth and sporulation of the parent *B. cereus* were normal, but mutant No. 52 was always nonsporulating, although displaying a similar growth pattern. Since the other two temperature-sensitive mutants, Nos. 40 and 54, also performed in a similar fashion as did mutant No. 52, only one mutant will be reported on in detail.

The sporogenic cultures (Figs. 1, 2, 3) utilized zinc during growth and sporulation. In regard to the normal *B. cereus* cultures grown at 37° C (Fig. 3), although zinc was utilized during growth and sporulation, release of zinc did occur by the end of the spore cycle. Normal *B. cereus* cultures grown at 37° C

TABLE I

Chemical changes occurring in a minimal synthetic medium during growth and sporulation of normal *B. cereus* cells and of *B. cereus* mutant No. 52 at 28° C

	0 hr	18 hr	30 hr	40 hr	54 hr
	Normal *B. cereus* cells				
Glucose (mg/ml)	6	4.8	2.2	0.2	0.2
Diaminopimelic acid (+ or −)*	−	+	+	+	+
Dipicolinic acid (+ or −)*	−	−	−	−	+
Total nitrogen (μg/ml)	950	224	200	189	186
Ammonia nitrogen (μg/ml)	0	3	3	25	45
Amide nitrogen (μg/ml)	0	0	1	5	10
Amino nitrogen (μg/ml)	950	209	194	133	83
Peptide nitrogen (μg/ml)†	0	12	2	26	48
Total phosphorus (μg/ml)	243	238	195	165	180
Organic phosphorus (μg/ml)	0	45	45	49	65
Inorganic phosphorus (μg/ml)	243	193	150	116	115
Potassium (μg/ml)	434	65	145	233	263
Iron (μg/ml)	2.8	0.9	2.1	2.2	2.6
Magnesium (μg/ml)	20	15.4	13.0	2.7	1.9
	B. cereus mutant No. 52				
Glucose (mg/ml)	6	0.8	0.3	0.2	0.2
Diaminopimelic acid (+ or −)*	−	+	+	+	+
Dipicolinic acid (+ or −)*	−	−	−	−	+
Total nitrogen (μg/ml)	950	176	140	140	158
Ammonia nitrogen (μg/ml)	0	8	40	56	63
Amide nitrogen (μg/ml)	0	2	6	3	5
Amino nitrogen (μg/ml)	950	118	84	55	22
Peptide nitrogen (μg/ml)†	0	48	10	26	68
Total phosphorus (μg/ml)	243	192	138	152	170
Organic phosphorus (μg/ml)	0	52	24	58	59
Inorganic phosphorus (μg/ml)	243	140	114	94	111
Potassium (μg/ml)	434	213	238	275	388
Iron (μg/ml)	2.8	1.7	2.5	3.0	2.2
Magnesium (μg/ml)	20	10.4	1.8	0.8	1.1

*+ = present; − = absent.
†Residual nitrogen.

released cellular manganese into the medium after 14 hours of incubation, with subsequent reutilization during sporulation. However, when cultured at 28° C, the same parent *B. cereus* consumed all the manganese during growth, with some release as spores were formed. When mutant No. 52 cells were incubated at 28° C, manganese uptake occurred during growth, with most of the manganese being returned to the medium by the end of spore formation.

TABLE II

Chemical changes occurring in a minimal synthetic medium during growth and sporulation of normal *B. cereus* cells and during growth and "abortive" sporulation of *B. cereus* mutant No. 52 at 37° C

	0 hr	14 hr	18 hr	24 hr	30 hr
Normal *B. cereus* cells					
Glucose (mg/ml)	6	3.1	0.4	0.3	0.2
Diaminopimelic acid (+ or −)*	−	+	+	+	+
Dipicolinic acid (+ or −)*	−	−	−	−	+
Total nitrogen (μg/ml)	950	270	186	165	150
Ammonia nitrogen (μg/ml)	0	2	4	20	33
Amide nitrogen (μg/ml)	0	1	5	11	11
Amino nitrogen (μg/ml)	950	203	171	121	106
Peptide nitrogen (μg/ml)†	0	64	6	13	0
Total phosphorus (μg/ml)	243	215	172	185	195
Organic phosphorus (μg/ml)	0	10	7	0	7
Inorganic phosphorus (μg/ml)	243	205	165	188	188
Potassium (μg/ml)	434	213	295	385	313
Iron (μg/ml)	2.8	0.1	1.6	2.5	3.0
Magnesium (μg/ml)	20	16.5	15.3	7.0	3.8
B. cereus mutant No. 52					
Glucose (mg/ml)	6	1.6	0.6	0.4	0.2
Diaminopimelic acid (+ or −)*	−	+	+	+	+
Dipicolinic acid (+ or −)*	−	−	−	−	+
Total nitrogen (μg/ml)	950	214	199	143	160
Ammonia nitrogen (μg/ml)	0	7	14	40	52
Amide nitrogen (μg/ml)	0	1	18	22	17
Amino nitrogen (μg/ml)	950	206	154	53	87
Peptide nitrogen (μg/ml)†	0	0	13	28	4
Total phosphorus (μg/ml)	243	185	200	188	165
Organic phosphorus (μg/ml)	0	32	54	53	55
Inorganic phosphorus (μg/ml)	243	153	146	135	110
Potassium (μg/ml)	434	135	240	233	220
Iron (μg/ml)	2.8	0.8	1.2	3.5	3.6
Magnesium (μg/ml)	20	13.8	12.4	0.8	0

*+ = present; − = absent.
†Residual nitrogen.

All three mutants when asporogenic (Fig. 4, mutant No. 52 specifically) displayed a difference from sporogenic cultures in the utilization of zinc and manganese. Although zinc was metabolized by the three mutants during growth, it appeared not to be consumed from the 18th hour on; contrarily, it was released into the medium. The difference between sporogenic and asporogenic cultures regarding zinc metabolism is certainly not striking (Figs. 3 and 4), but has been included because of an earlier observation noted with zinc. This will be subsequently discussed. Manganese, on the other hand, was utilized during the first 14 hours of growth, and then was completely consumed during "abortive" sporulation.

The remainder of the medium components and metabolic products analyzed are shown in Tables I and II. Differences in these between sporogenic and asporogenic cultures were either absent or only of a minor nature. The bulk of glucose and amino-N was utilized during growth, with little being used during the spore or "abortive" spore phase. Inorganic-P, potassium, iron, and magnesium were consumed during growth and/or sporulation or during growth and/or "abortive" sporulation. Diaminopimelic acid accumulated in the medium throughout the entire culture cycle. Only traces of dipicolinic acid were found in the media of both sporogenic and nonsporogenic cultures. The presence of dipicolinic acid was not detected until the completion of the culture cycle had occurred at both 37° C and 28° C. Generally, a steady increase of both ammonia-N and amide-N was noted in the medium, with ammonia-N levels being somewhat higher. Both peptide-N and organic-P were found in the medium of sporogenic and asporogenic cultures.

Discussion

The results of chemical analyses revealed differences in the way some of the medium components were utilized by the parent and mutant cultures of *B. cereus*. The observed changes in the level of zinc at different stages of the 30-hour growth–sporulation cycle (37° C) indicate a possible variation in response by sporulating and nonsporulating cultures. Some uptake of zinc, while growth and sporulation progressed, occurred in sporulating cultures, whereas zinc consumption apparently stopped in cultures undergoing "abortive" sporulation. In the development of the minimal medium, zinc had been demonstrated to be essential for sporulation (11). At 37° C, zinc utilization by the three mutants appeared to be inhibited; this inhibition may be part of the reason why sporulation was "abortive". How this inhibition of zinc uptake is related to the temperature-sensitivity aspects of asporogenesis should prove most interesting. More than likely the role of this metal is as a cofactor for some enzyme system(s). Dixon and Webb (4) list at least seven enzymes which employ zinc as a cofactor, including carboxypeptidase and L-glutamate dehydrogenase. Gollakota and Halvorson (7) have shown that α-picolinic acid specifically inhibited sporulation of *B. cereus* without causing permanent injury to the organism. The inhibition could be reversed by zinc as well as some other metals and yeast extract. These authors postulated that α-picolinic acid interferes (by chelating with some metal) with the formation of an enzyme system adaptively formed between the logarithmic growth phase and the stationary phase. This enzyme system apparently is concerned with the metabolism of acid intermediates accumulating in the medium during growth.

The metabolism of manganese from the exogenous environment was also somewhat different by mutant cultures when grown under conditions leading to asporogenesis. During "abortive" sporulation the mutants consumed all the manganese from the medium, which was not the case when sporulation occurred. The role of this cation is again probably as a cofactor, which requirement is minimal during sporulation. The importance of metal ions was earlier anticipated by Foster and Heiligman (6), who maintained that they supply the proper enzyme balance necessary for spore formation. By using mutants that have different responses to cation requirements for sporulation, one may

eventually uncover specific enzyme systems (and reactions) directly involved in sporulation.

Special interest was noted from the results of some of the metabolic by-products accumulating during growth and sporulation; in particular, the release into the medium of both diaminopimelic acid and dipicolinic acid (DPA). Diaminopimelic acid was not quantitated and therefore no complete assessment was possible. Dipicolinic acid has only been found in cells undergoing sporulation and not in any other place in the biological world (15, 16), signifying some unique metabolic role in the spore. Earlier observations by Powell and Strange (16) demonstrated that DPA was unique to spores or cells in the final stages of spore maturation. The results in this report show some DPA to be present in the medium of sporulating cultures at the end of the spore cycle. Some DPA was even found in the medium of nonsporulating cultures at the end of the "abortive" spore phase. However, there was essentially no difference between normal and "abortive" sporulation in the amounts of DPA found in the medium. Powell and Strange showed that vegetative cells and cells in the early stages of spore formation contained no DPA. However, although Powell and Strange analyzed the medium during growth and sporulation, there was no specific mention of DPA. To the authors' knowledge, there has not as yet appeared any published literature pertaining to the presence or absence of DPA in culture media of sporeforming cells. Microscopic observations of both stained and viable cells taken during different culture phases revealed no apparent evidence of cell lysis to account for the medium DPA. Further, the amino-N level of the medium decreased consistently with time, which probably would not have happened if cell lysis had occurred. There is, though, a noticeable morphological difference in the normal culture and asporogenic mutants. A final explanation of the DPA situation in this *B. cereus* culture must wait until additional evidence is available.

The results of iron utilization during growth and its release into the medium during the cultural sporulation phase are, however, contrary to the findings of Powell and Strange (16). Powell and Strange found that the iron content of cells rose during spore formation. The results reported here indicate a fall in the iron content of the medium between 14 hours and 18 hours, with a subsequent rise during sporulation. These apparent differences are probably best explained by pointing out that the strain of *B. cereus* investigated may be inherently different from those of the other investigators. Also, the medium and cultural conditions employed here were different; it is not at all improbable that these chemical and physical differences could affect the cells' metabolic capacities.

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THE METABOLIC DETOXICATION OF 2,4-DINITROPHENOL BY FUSARIUM OXYSPORUM[1]

CLARENCE MADHOSINGH[2]

Abstract

Dinitrophenol (DNP) was found to be reduced to the less toxic 2-amino-4-nitrophenol and its isomer 4-amino-2-nitrophenol by *Fusarium oxysporum* (Schl.) em. Snyder and Hansen. There were indications that these compounds can also be altered by the organism.

The greatest reduction of DNP occurred during the first 20 days of incubation at pH 7.1 under the conditions of the experiments. The growth of *F. oxysporum* and the reduction of DNP were stimulated by sodium nitrate, increasing to a concentration of 1.2%. *F. oxysporum* produced 2,4-dinitrophenol in media treated with synthetic 2-amino-4-nitrophenol, indicating the presence of a reversible nitro-reducing system in this organism.

The possible effect of the reduction of DNP on the invasion of treated wood by wood-rotting fungi is suggested and the significance of this in the problem of wood-preservation is indicated.

Introduction

Most of the information concerning the tolerance of individual fungi to 2,4-dinitrophenol (DNP), or to mixtures containing this substance, deals with organisms of direct economic importance. DNP is used extensively in fungicidal preparations especially for the preservation of timber in the railroad, mining, and telephone industries. The effect of associated organisms under natural conditions is a factor which should be considered in the critical evaluation of the fungicidal capacity of all preservatives. These organisms may modify the physiology of the prime organism or the toxic capacity of the preservative substances to such an extent as to render the treatment ineffective.

The metabolic reduction of DNP by mammalian tissue homogenates has been recorded by several workers (5, 11, 14). No report has been found in the literature, however, concerning the reduction of this compound by fungi.

A preliminary study (9) has shown that *Fusarium oxysporum* was more tolerant of DNP than some other wood-decaying fungi such as *Coprinus micaceus*.

In addition, it was found that the toxicity of preservatives was determined by the concentration of DNP in the mixture. This compound was always present in much lower molar concentrations than the other components. The same concentrations of DNP when used alone produced similar toxic effects. In all treatments involving DNP, *F. oxysporum* was much more tolerant than was *C. micaceus*.

It was concluded from these findings that the *Fusarium* organism was

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probably capable of reducing the toxicity of the DNP and thereby facilitated the infestation and spread of *C. micaceus*, which was found to be associated with *F. oxysporum* in decayed fence posts.

The object of this study was to investigate the fate of DNP in cultures of *F. oxysporum*.

Materials and Methods

Cultural Methods

A basal liquid medium was used for all cultures, except when indicated otherwise. This basal medium consisted of salts and sugar in tap water in the following concentrations, expressed as percentages: sodium nitrate, 0.2; potassium dihydrogen phosphate, 0.1; potassium chloride, 0.05; magnesium sulphate, 0.05; ferrous sulphate, 0.003; dextrose, 3.0.

Two buffer systems were used in the media. A phosphate buffer was used in studies undertaken between pH 7 and pH 8. When a greater range of hydrogen ion concentration was required a citric acid – phosphate buffer system was used. Solutions of buffers, of salts, and of sugar, respectively, were sterilized by autoclaving at 15 p.s.i. and 248° F for 15 minutes. The solutions were then mixed and dispensed in 100-ml aliquots into 250-ml Erlenmeyer flasks under sterile conditions.

F. oxysporum (Schl.) em. Snyder and Hansen, and *C. micaceus* (Fr.), the two organisms used in the experiments, were isolated and are maintained at the Forest Products Laboratory at Ottawa. *F. oxysporum* was identified by Dr. W. L. Gordon of the Canada Department of Agriculture at Winnipeg.

The inocula, in all cases, consisted of 0.6-cm-diameter disks obtained from the periphery of 6- to 10-day cultures on malt agar. The inoculated media were incubated in flasks at 27° C and 80% relative humidity in a dark environment. At the end of the incubation period, the cultures were filtered through Whatman No. 1 filter paper.

Each experiment consisted of a number of treatments each of which contained a different concentration of the preservative chemical. Each treatment was made up of four flasks. Two of these were used for analytical purposes, while the remaining two were used for hydrogen ion and mycelial weight determinations. Four control cultures, containing no preservatives, were included in each experiment. They served for comparison of pH and mycelial weight as well as for analytical controls.

Enzymic action was stopped by the addition of 5 ml of 50% (weight/volume) trichloroacetic acid per 100 ml of filtered medium.

Chromatographic examination of media treated with DNP did not indicate the presence of new compounds after 50 days under incubation conditions. The DNP content of the media remained the same throughout this period.

Chemical Methods

The analytical procedure for the separation and identification of DNP and its metabolic derivatives closely followed the method described by Guebert (7).

Chromatographic Methods

Chromatographic studies were undertaken as an aid to identification of

and as a means of separating the various metabolic by-products of the phenolic compounds in the residual medium. Whatman No. 1 paper was used in three solvent systems. Known synthetic compounds were used as standards in the various systems for comparison with the metabolites.

An ethyl ether extract from the medium removed the DNP and the aminonitrophenols. The aqueous residue was either evaporated to near dryness or applied directly to the chromatograms. The R_f values recorded for each compound were the averages of values obtained in quadruplicate in each solvent system.

The Separation and Estimation of Metabolic Products

The compounds were separated on Whatman No. 3 MM paper using solvent A (see Table I). An ether extract, obtained from the acidified residual medium, was evaporated slowly *in vacuo* over warm water to a final volume of about 1 ml. This was done using a 50-ml suction flask wrapped in aluminum foil in order to exclude as much light as possible. It is important that the ether extract should not contain water, which greatly delays the application of the material to the paper and facilitates the decomposition of the compounds by the action of both light and oxygen. The solvent was contained in a desiccator from which the internal supporting grill was removed. The desiccator, which had a vacuum outlet in the lid, was enclosed in aluminum foil during the separations. The system was evacuated immediately after the "spotted" chromatogram was inserted and the separation was allowed to proceed under reduced pressure (25 in. Hg) away from light and at room temperatures (23–25° C) for a period of about 90 minutes.

No developing agent was necessary for identifying the positions of the separated products since their respective bands appeared distinctly in characteristic colors under the alkaline conditions of the system. The separated compounds were extracted immediately (8) or stored for short periods in an evacuated desiccator in the dark. The elution fluid consisted of a 5% ammonia solution. Estimations of the "ether-insoluble" aqueous metabolites were made on a visual basis.

Spectrophotometric Methods

The Beckman DU spectrophotometer was used for quantitative estimations of the phenolic compounds. The DNP was determined by its absorption at 400 mμ in 5% ammonia (8). The aminonitrophenols were diazotized and coupled with β-naphthol (7) and determined colorimetrically (8).

Results

Reduction Products of 2,4-Dinitrophenol

Chemical analyses of the DNP-treated media (buffered at pH 7.2), which had maintained the growth of *F. oxysporum*, indicated the presence of DNP and newly formed 2-amino-4-nitrophenol and the isomer 4-amino-2-nitrophenol. Chromatographic studies of the ether extract from media which were treated similarly (Tables II, III) provided further evidence in support of the formation of these compounds in the presence of the growing organism, since the R_f values for these metabolites were the same as those of the synthetic standards (Table I). In addition, these studies indicated the occurrence of

another compound, an unknown, which appeared in the ether extract of DNP-treated cultures of *F. oxysporum* that were between 15 and 30 days old. This substance had an R_f value of 0.44 (solvent A) and appeared between the two aminonitrophenols. This value could not be identified with those of several other phenols (Table I). The aqueous residue (after ether extraction) of DNP-treated *F. oxysporum* cultures produced three spots on the chromatogram in solvent A with R_f values of 0.25, 0.1, and 0.0 (Table II). When this solution was reduced to near-dryness by evaporation from dialyzing tubing in air, and this concentrated residue applied to the chromatogram, there was no migration of the material on the paper.

C. micaceus, grown under similar conditions at concentrations of DNP ranging from 0.001% to 0.006%, gave no indication of any reduction products in either chromatographic studies (Table II) or chemical analyses.

The Effect of Time on the Reduction of DNP

The amount of DNP which was metabolized by *F. oxysporum* increased with time, the rate being rapid during the first 20 days and then gradually becoming slower between the 20th and 30th days (Fig. 1). The medium initially contained 32.6 μmoles DNP per 100 ml buffered at pH 7.2. Interestingly, a 50-day-old treated culture of the organism contained 8.40 μmoles of DNP as compared with 4.48 μmoles in 30-day-old cultures incubated under

ABBREVIATIONS USED IN TABLES ON CHROMATOGRAPHY (TABLES I TO III)

Chromatographic Solvent Systems

A. Benzene 20%
Pentanol-1 20%
Ethanol 40%
Ammonia solution (sp. gr. 0.88) 20%
Used in ascending chromatography under reduced pressure.

B. Benzene: glacial acetic acid: water in a 1:1:2 volume-to-volume mixture. The organic phase was used in descending systems.

C. 1-Butanol saturated with water was used as an ascending solvent.

Detecting Agents

1. Ultraviolet
2. Solvent A without further treatment
3. Solvent B without further treatment
4. Solvent C without further treatment
5. Silver nitrate 0.1 *N* in 5% NH_3 solution
6. 1 *N* sodium hydroxide

Color Code

a. Yellow
b. Orange yellow
c. Dark violet
d. Violet
e. Light blue
f. Fawn brown
g. Scorched paper brown-black
h. Flame red brown
i. Silver gray
j. Green gray
k. Brown gray
l. Pink
m. Orange

– Indicates no apparent color reaction with this detecting agent.

TABLE I

Paper chromatography of phenolic compounds

R_f ×100 for three solvent systems				Colors with various detecting agents					
C	B	A	Compounds	1	2	3	4	5	6
56	85	65	2,4-Dinitrophenol	c	a	a	a	–	b
86	100	52	2-Amino-4-nitrophenol	c	b		a		b
80	100	40	4-Amino-2-nitrophenol	d	f	f	f		l
12		30	2,4-Diaminophenol	h	h	–	f	g	f
84	60	36	*p*-Nitrophenol	e	a	a	–	b	a
84	60	80	*m*-Nitrophenol	–	–			b	m
84	87	56	*o*-Nitrophenol	c	–		–	–	b
72	5	71	*p*-Aminophenol	e	f		f	g	f
70	22	72	*m*-Aminophenol	e	–		–	g	f
52		70	Picric acid	c	a		a	–	b –
	6	30	Picramic acid	c		–		g	–
80	83	87	Picramide		a	a			
30		66	Aniline	e		–		f	
73	15	73	Resorcinol	e				g	k
91	100	88	Dye of 2-amino-4-nitrophenol*	–	l		l		l
90	100	74	Dye of 4-amino-2-nitrophenol*	d	f		f		l
			2,4-Diaminophenol–HCl†						
12		29	Spot 1		b		l		f
3		20	Spot 2	h	f		l		–
0	0	10	Spot 3		k		g		g

*Diazotized and coupled with β-naphthol.
†Solution chromatographed after standing for 72 hours.

TABLE II

Chromatography of extracts from 2,4-dinitrophenol-treated cultures of *F. oxysporum* and *C. micaceus*

$R_f \times 100$ for three solvent systems			"Spotting" material	Colors with various detecting agents						Identity of spots
C	B	A		1	2	3	4	5	6	
F. oxysporum 20-day-old culture treated with 32.6 μmoles DNP										
			Ether extract							
55	85	65	Spot 1	c	a	a	a	—	b	DNP
87	100	52	Spot 2	c	b		a		b	2-Amino-4-nitrophenol
90		44	Spot 3	—	a	—		b	g	
81	100	40	Spot 4	d	f		f		l	4-Amino-2-nitrophenol
12		25	Spot 5	h	f		f	g	f	
			Aqueous residue							
12		25	Spot 6	h	f		l		f	
3	0	10	Spot 7	d	f		l		—	
0	0	0	Spot 8	d	k		g		f	
Control—20-day-old medium treated with 32.6 μmoles DNP, not inoculated										
			Ether extract							
55	86	66	Spot 9	c	a	a	a	—	b	DNP
—	—	—	*Aqueous residue*	—	—	—	—	—	—	
C. micaceus 20-day-old culture treated with 5.4 μmoles DNP (Mycelial weight: 64 mg)										
			Ether extract							
56	85	64	Spot 10	c	a	a	a	—	b	DNP
—	—	—	*Aqueous residue*	—	—	—	—	—	—	

TABLE III

Chromatography of extracts from cultures of *F. oxysporum* treated with aminonitrophenols

$R_f \times 100$ for three solvent systems			"Spotting" material	Colors with various detecting agents						Identity of spots
C	B	A		1	2	3	4	5	6	
F. oxysporum 12-day-old culture treated with 65 μmoles 2-amino-4-nitrophenol										
			Ether extract							
56	84	64	Spot 1	c	a	a	a	—	b	DNP
86	100	52	Spot 2	c	b		a		b	2-Amino-4-nitrophenol
11		27	Spot 3		a		l			
			Aqueous residue							
12		20	Spot 4		a		l			
Control—12-day-old medium treated with 65 μmoles 2-amino-4-nitrophenol, not inoculated										
			Ether extract							
86	100	52	Spot 5	c	b		a	b		2-Amino-4-nitrophenol
—	—	—	*Aqueous residue*		—	—	—			
F. oxysporum 12-day-old culture treated with 65 μmoles 4-amino-2-nitrophenol										
			Ether extract							
90		45	Spot 6	—	a		—	b	g	
81	100	41	Spot 7	d	f	—	f		l	4-Amino-2-nitrophenol
11		28	Spot 8		f		l		f	
		18	Spot 9		a					
			Aqueous residue							
10		29	Spot 10		f		l		f	
		10	Spot 11		f					
Control—12-day-old medium treated with 65 μmoles 4-amino-2-nitrophenol, not inoculated										
			Ether extract							
80	100	42	Spot 12		f	—	f		l	4-Amino-2-nitrophenol
12		25	Spot 13		f		l		f	
		7	Spot 14		l	—	f		l	

similar conditions. The amount of 2-amino-4-nitrophenol present in the medium closely paralleled the amount of dinitrophenol altered. The rate of increase was more rapid during the early period of growth, gradually decreasing after the 15th day (Fig. 2). Only 7.8 μmoles of this metabolite were present in the 50-day-old treated culture as compared with 9.22 μmoles in the 30-day-old cultures.

The formation of 4-amino-2-nitrophenol was initially slow and the ratio of the amount of this compound to the amount of the 2-amino isomer showed,

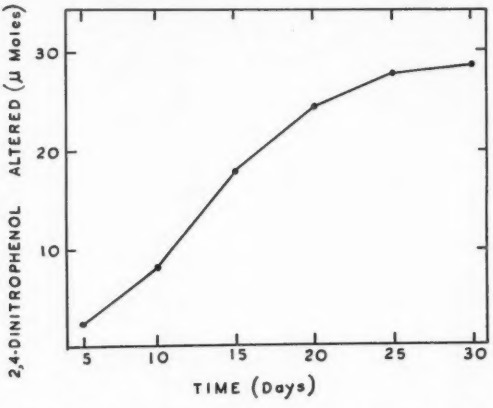


FIG. 1. The effect of time on the alteration of DNP by *F. oxysporum*.

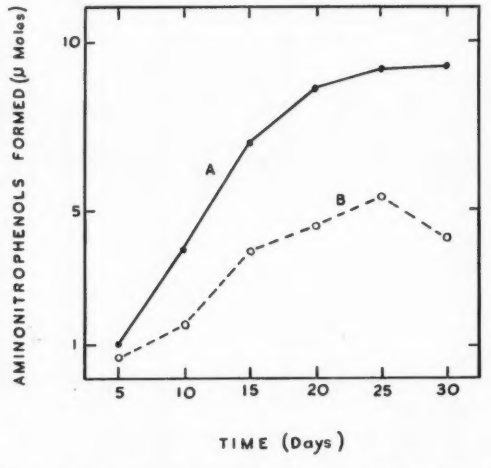


FIG. 2. The effect of time on the formation of: A, 2-amino-4-nitrophenol, and B, 4-amino-2-nitrophenol, in cultures of *F. oxysporum* treated with DNP (32.6 μmoles/100 ml medium).

from the 15th day, a decrease which became especially marked after the 25th day (Fig. 2). Extracts from the 50-day-old cultures showed no signs of this compound on the chromatograms. The molar concentration of 2-amino-4-nitrophenol was always higher than that of the 4-amino isomer.

By visual estimation the amount of water-soluble amines in the residual media showed a rapid increase especially after the 25th day and the increase was quite noticeable in the 50-day-old cultures. The unknown compound did not appear on the chromatograms of the ether extract from the 5th- and 10th-day samples. This substance appeared as a yellow band on the 15th day, and the intensity of the color was at a maximum between the 25th and 30th days. There were no indications of this material in the ether extracts from 50-day cultures.

The Effect of pH on the Reduction of 2,4-Dinitrophenol

The optimum pH for the metabolism of DNP was found to be pH 7.1 under the conditions of the experiment. There was a decrease in the more acid and alkaline media but especially in the former (Fig. 3). It will be noted, however, that the optimum pH for growth, recorded in terms of mycelium weight, was at pH 8. Under the alkaline conditions, the relationship between mycelium weight and the amount of DNP altered was less consistent than under acid conditions.

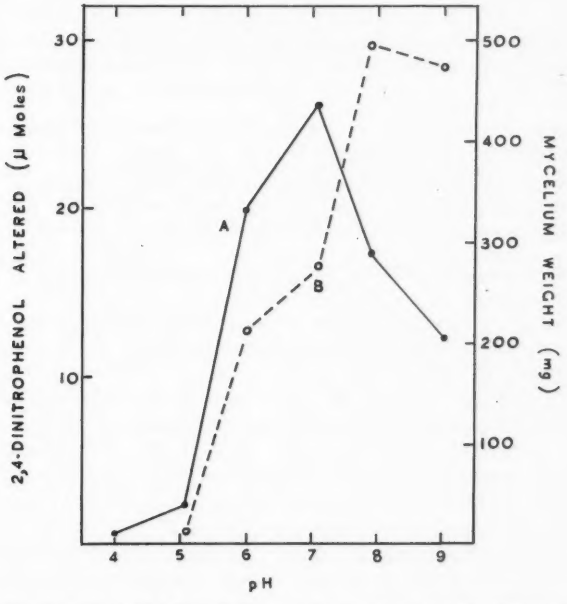


FIG. 3. A comparison of the effect of pH on: A, the alteration of DNP, and B, mycelium weight (18-day cultures).

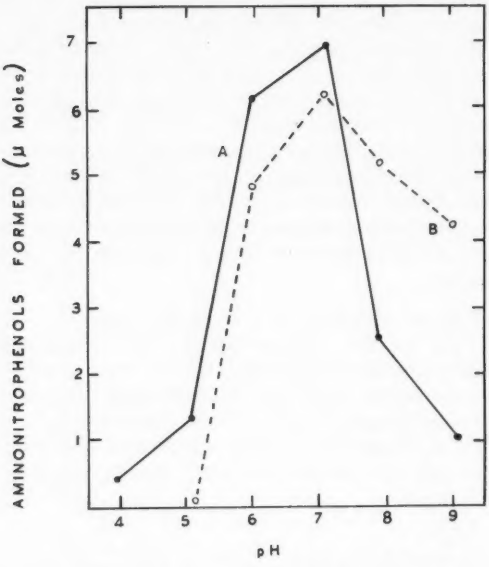


FIG. 4. The effect of pH on the formation of amines in 18-day cultures of *F. oxysporum* treated with 32.6 μmoles DNP/100 ml medium: A, 4-amino-2-nitrophenol; B, 2-amino-4-nitrophenol.

At the lower pH values there was a greater amount of the 4-aminonitrophenol than the 2-amino isomer whereas the reverse was the case in the alkaline cultures (Fig. 4). The amount of the "ether-insoluble" compounds in the aqueous residue and of the unknown compound on the chromatograms appeared to be greatest between pH 7 and pH 8.

The Effect of Sodium Nitrate Concentration on the Reduction of 2,4-Dinitrophenol

The growth of *F. oxysporum* was stimulated by sodium nitrate, increasing to a concentration of 1.2%. The organism attained the most growth and accomplished the highest percentage reduction of DNP at this concentration of the salt. The cultures grew for 17 days to a weight of 730 mg in media treated with 32.6 μmoles DNP. This concentration of sodium nitrate was higher than that which was normally used in the basal medium. Higher concentrations of the nitrate salt affected adversely both the ability of the organism to alter the toxic compound and also its growth (Table IV).

The amount of the metabolites was especially high in this experiment, with the 2-amino-4-nitrophenol being present in predominantly greater quantities than the 4-amino isomer. These compounds, together with the "ether-insoluble" and the unknown compounds, were in maximum amounts at the 1.2% concentration of the nitrate salt (Table IV).

TABLE IV

The effect of various concentrations of sodium nitrate on the alteration of 2,4-dinitrophenol (Cultures 17 days old, buffered at pH 7.2. Flasks contained 32.6 μmoles DNP in 100 cc medium)

Concn. of sodium nitrate (%)	Mycelium weight (mg)	Residual DNP (μmoles)	DNP altered, calc. (μmoles)	2-Amino-4 nitrophenol, amt. present (μmoles)	4-Amino-2 nitrophenol, amt. present (μmoles)
0.0	62	23.10	9.50	4.52	2.61
0.4	610	12.02	20.58	5.51	6.12
0.8	677	8.48	24.12	6.35	6.51
1.2	730	4.80	27.80	9.25	7.21
1.6	579	7.39	25.21	9.21	7.13
2.0	540	11.45	21.15	8.05	6.19
0.4*	1045	—	—	—	—

*No DNP in media.

Growth of F. oxysporum and C. micaceus in the Presence of Synthetic Nitrophenols

Growth of *F. oxysporum* in various concentrations of the 2-amino-4-nitro compound in the basal medium containing 0.008% ferrous sulphate produced rather interesting results. Chromatography of ether extracts obtained from 12-day treated cultures using solvent A showed the presence of two new compounds. One position corresponded exactly to that of DNP (Table III). This metabolite was eluted and in chromatography gave R_f values identical with those of synthetic DNP. The formation of DNP at various concentrations of 2-amino-4-nitrophenol is shown in Table V. Control medium, which was not inoculated, showed no new compounds.

TABLE V

The formation of dinitrophenol by *F. oxysporum* at various concentrations of 2-amino-4-nitrophenol (Culture 12 days old, basal medium containing 0.008% $FeSO_4$)

Amount of aminonitrophenol in 100 cc of medium (μmoles)	Mycelium weight (mg)	Amount of dinitrophenol formed (μmoles)
26	398	4.10
39	406	5.66
52	514	6.24
65	583	6.60
130	336	3.82
195	220	3.68
260	115	2.94
390	90	—
0.0	176	—

Concentrations of 2-amino-4-nitrophenol ranging from 26 to 195 μmoles induced an appreciable stimulation in the growth of the organism above that in the untreated control flasks.

Chromatographic studies with the extracts obtained from cultures of *F. oxysporum* treated with 4-amino-2-nitrophenol showed the presence of three

new compounds (Table III). The R_f value of 0.46 for the metabolite compared favorably with the value obtained for the ether-soluble unknown compound (Spot 3, Table II). Only the lowest concentration (0.0006%) of this compound had any stimulatory effect on the growth of the fungus. Growth was greatly limited by the presence of this substance in treatments at higher concentrations. There was no growth by the organism at the 0.08% concentration of either the 4-amino-2-nitrophenol or the 2-amino-4-nitrophenol.

In order to determine the comparative toxicity of DNP, 2-amino-4-nitrophenol, and 4-amino-2-nitrophenol, the two organisms were grown in concentrations of these compounds ranging from 1 μmole to 500 μmoles in 100 ml malt extract (1.25%) contained in 250-ml flasks. There were four replicates in each treatment and cultures were grown at room temperatures (22–25° C). After 10 days' incubation the mycelia were filtered and dried to constant weight at 60° C.

The weights of the mycelia of both fungi were higher when grown in medium containing the aminonitrophenols than in medium containing DNP, indicating the greater toxicity of DNP (Fig. 5). *F. oxysporum* was more tolerant than *C. micaceus* to all the treatments. In Table VI the lethal concentration refers to the concentration of the toxic compound in which the inocula were killed after 10 days.

The lethal dose of DNP for *F. oxysporum* was six times greater than that

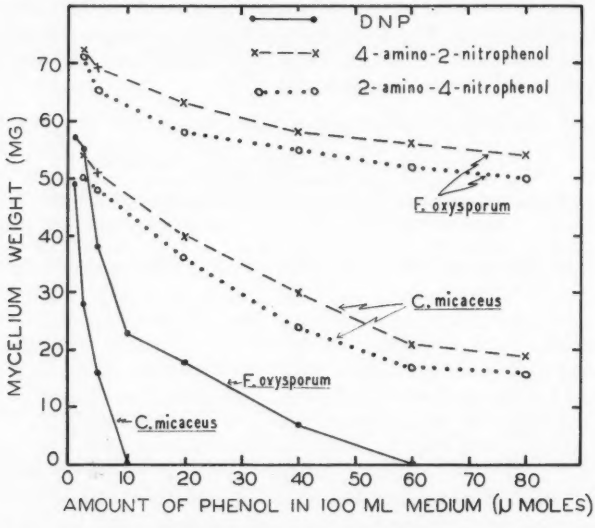


FIG. 5. Comparative toxicity of DNP, 2-amino-4-nitrophenol, and 4-amino-2-nitrophenol to *F. oxysporum* and *C. micaceus*. Mycelium weight values are the averages of four 10-day cultures which were grown at room temperatures in 100 ml malt extract (1.25%). The mycelium was filtered and dried to constant weight at 60° C. The mycelium weights from cultures of *F. oxysporum* and *C. micaceus* grown in nontreated medium were 62 mg and 54 mg respectively.

TABLE VI

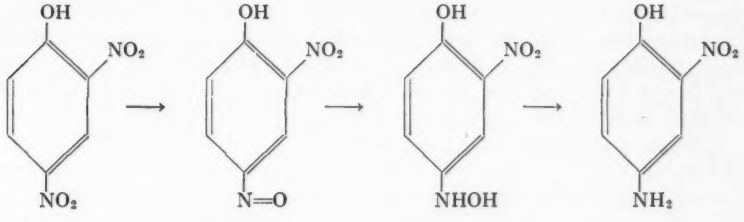
Lethal concentrations of phenolic compounds to *F. oxysporum* and *C. micaceus* (μmoles/100 ml medium)

	DNP	2-Amino-4-nitrophenol	4-Amino-2-nitrophenol
F. oxysporum	60	over 500	400
C. micaceus	10	300	300

for *C. micaceus*. This difference in lethal dosage was greatly reduced, however, when the organisms grew in the presence of the aminonitrophenols (Table VI).

Discussion and Conclusion

The results obtained from experiments carried out in this study have shown that DNP was reduced by the organism *F. oxysporum* to 2-amino-4-nitrophenol and 4-amino-2-nitrophenol. This reduction of the nitro groups on the benzene ring might be postulated to take place in stages, involving the intermediate formation of the nitroso and hydroxyamino groups. Thus, the reduction of the nitro group in the para position of the ring would probably be as follows:



Dinitrophenol — 4-Nitroso-2-nitrophenol — 4-Hydroxyamino-2-nitrophenol — 4-Amino-2-nitrophenol

The 2-amino-4-nitrophenol isomer is thought to have been formed similarly by reduction of the nitro group in position 2.

The experiments of Neuberg and Welde in 1914 (10) indicate quite strongly the possibility of this three-step reduction of the nitro group to the amine in biological systems. On the other hand, the data obtained by Yamashina *et al.* (15) showed that the nitroso compound was the direct precursor of the amine when aromatic nitro compounds were reduced by certain bacteria, and that the hydroxyamino derivative was by-passed in the reduction.

Experiments, in which *F. oxysporum* was grown in media treated with the synthetic aminonitrophenols, have thrown some light on the probable course of the alteration of dinitrophenol. The yellow band (R_f 0.44 in solvent A) which appeared on chromotagrams of the ether extract of 4-amino-2-nitrophenol-treated cultures of *F. oxysporum* was believed to be the same metabolite as the unknown compound which was extracted with ether from dinitrophenol-treated cultures. This substance was not present in the ether extracts of 2-amino-4-nitrophenol-treated cultures. These facts present the possibility

that the unknown compound may not have been a direct product of DNP metabolism but one actually resulting from the breakdown of the 4-amino-2-nitrophenol.

The formation of DNP in cultures treated with 2-amino-4-nitrophenol indicated the likelihood of a reversible system being present in the organism. The oxidation of aminonitrophenol appears to be unique in a biological system as no report has been found concerning this. Granville and Stern (6), who used various enzymes and redox indicator systems in *in vitro* studies, were unsuccessful in their attempts to obtain a condition suitable for the reversal of the reduction reaction of DNP.

This formation of DNP from the 2-amino derivative by *F. oxysporum* probably accounts for the persistent residue of the dinitro compound in cultures treated with this substance, since an equilibrium was probably maintained between the concentration of DNP and the concentration of the amino derivatives in the cultures.

The results of other workers (1, 3, 4) give some indication of the types of compounds which one could expect beyond the aminonitrophenol stage of reduction.

It was not surprising that the pH of the medium played such an important part in the reduction of DNP. Since the poison was reduced by the growing organism, it could be expected that the reduction would be proportional to the amount of living mycelium present in the cultures. The reduction increased to an optimum at pH 7.2. The highest weight of mycelium was, however, nearer to pH 8. The greater disparity between growth and reduction of DNP in alkaline conditions indicates the influence of pH in differentiating these two metabolic systems.

The enzyme systems obtained form mammalian tissues and bacterial cells all demonstrated maximum efficiency in the reduction of aromatic nitro compounds, including DNP, between pH 7 and pH 7.5 (5, 11, 12, 13, 14).

Figure 4 shows that the 4-aminonitrophenol compound was found in excess of the 2-amino isomer in the acid cultures whereas there was a sharp reversal in the proportions at the higher pHs. This led to the speculation that the 4-amino compound was reduced to a greater extent under alkaline conditions than was the 2-aminonitrophenol. This seems probable because of the increase in the amount of secondary breakdown products in the aqueous residue. Also there was only a comparatively small difference in the proportion between the DNP altered and the formation of the 2-amino isomer in the more basic cultures.

The levelling off of the curve for the metabolism of DNP after the 25th day (Fig. 1) was probably a sign that a certain equilibrium had been attained between the concentrations of the various metabolites in the system as a whole. The relative divergence from one another of the curves for the amount of the 2-amino and 4-amino nitro compounds present was probably due to the 4-amino isomer alteration which appeared very pronounced after the 25th day. The complete disappearance of the 4-amino compound from the ether extract of the 50-day treated culture could possibly be accounted for by the increase in the contents of the aqueous residue. The slight increase in

the amount of DNP was probably due to the oxidation of some of the metabolic 2-aminonitrophenol which was present in the younger cultures.

Basically, the study has indicated reasons for the complementary action of two microorganisms which resulted in the decay of treated wood (8). Under natural conditions, the *F. oxysporum* would grow into DNP-treated fence posts and reduce the poison to less toxic compounds thereby permitting the growth of the *Coprinus* spp. The findings also point to the possibility of a nitro reductase system in the *Fusarium* fungus similar to or at least comparable with that observed in mammals. Furthermore, there have been indications of the presence of a reversible system for the oxidation-reduction of aromatic nitro compounds. A report of such a system has not been found in the literature.

Acknowledgments

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GROWTH STUDIES ON ARTHROBACTER GLOBIFORMIS[1]

I. L. STEVENSON

Abstract

Studies have been undertaken to establish the growth behavior of *A. globiformis* in liquid media of different nutritional levels. It has been found that this organism exhibits a distinct morphological pattern during the growth cycle. Cells reached maximum size just prior to division and at this time the pleomorphic forms typical of the genus *Arthrobacter* were observed. In a simple basal salts medium cells doubled in size while in a complex synthetic medium an 8- to 10-fold increase in cell size was noted during the predivision lag. After the lag period successive divisions occurred with a gradual decrease in size until the cells finally reverted to the normal coccoid form.

Introduction

In the general introduction to the genus *Arthrobacter* as given in Bergey's Manual (1) the member species are described as follows: "In young cultures the cells appear as rods which may vary in size and shape from straight to bent, curved, swollen or club-shaped forms. Short filament formation with rudimentary budding may occur, especially in richer media. Coccoid cells are characteristically observed in cultures after one or more days." It is evident from the literature pertaining to the morphology and growth habits of the *Arthrobacter* that the majority of the descriptions are based on sporadic observations of cells grown on solid or liquid media. Only a few investigators have reported on the cyclic nature of growth of these organisms with the suggestion that the pleomorphic characteristics of the cells were confined to specific stages of the growth cycle (2, 10, 12).

As a prelude to the study of the physiological aspects of growth throughout the life cycle of *Arthrobacter* spp. it was found necessary to investigate the growth patterns of these organisms in liquid cultures. The following studies are concerned with the behavior of the types species, *A. globiformis*, in a number of nutritional environments.

Materials and Methods

Growth studies on *A. globiformis* were performed in nepheloculture flasks containing 50 ml of the appropriate medium. The flasks were placed on a shaker operating in a 28° C water bath. Turbidimetric measurements were made at varying intervals, at which times appropriate samples were removed for plating, direct microscopic counts, and staining.

The media used in these studies consisted of the basal salts (B) and yeast-soil extract (YS) of Lochhead and Burton (9) as well as the complex synthetic media developed by Henderson and Snell (6).

Direct microscopic counts were made with a Helber counting chamber (cell depth 0.01 mm) using appropriate dilutions of the cultures. Plate counts

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were made by spreading 0.1 ml of serial dilutions on prepoured plates of solidified yeast-soil extract medium. Plates were incubated for 4 days at 28° C. Both methods were used for determining bacterial numbers in all experiments.

Stained specimens for photomicrographs were prepared as follows: Dilute suspensions of the organisms were spread on dried plates of 2% agar. Blocks were cut from the plates and exposed to osmium tetroxide vapors (over a 2% aqueous solution) for 2 minutes and inverted on clean glass slides. The smears were then stained with crystal violet (5%) for 30 to 45 seconds.

Turbidimetric measurements were made on a Klett colorimeter with a 540-mμ filter and readings expressed as Klett units. Calculated corrections were applied when turbidities exceeded the accurate range of the instrument.

Inocula for these studies consisted of 24-hour yeast-soil extract broth cultures of *A. globiformis*. The cultures were centrifuged (6000 r.p.m.), washed once and resuspended in distilled water, and brought to a Klett reading of 500 at 540 mμ. One milliliter of this suspension was used to inoculate 50 ml of media. In all studies the flasks were incubated in a shaker water bath at 28° C.

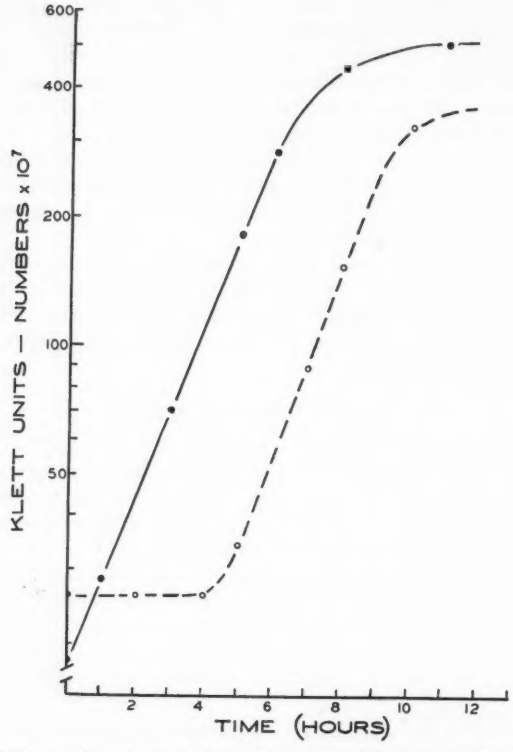

FIG. 1. Turbidity and numbers of *A. globiformis* growing in yeast-soil extract medium. Solid line, turbidity. Broken line, numbers of bacteria.

Results

Results of growth studies of *A. globiformis* in yeast-soil extract medium (YS) are presented in Fig. 1. Turbidimetric data indicate that cellular growth is initiated immediately and proceeds exponentially throughout the first 7 to 8 hours. Numbers of microorganisms, on the other hand, show a distinct lag period of 4 to 4.5 hours prior to actual division of the cells. After the lag, division proceeds exponentially. Microscopic examination has shown that during the initial lag period a five- to six-fold increase in cell size occurs accompanied by the morphological changes characteristic of the *Arthrobacter*. The photomicrographs in Figs. 4–9 illustrate changes of the organisms as they proceed through the growth cycle. Starting at 0 time as a typical coccoid cell (24-hour inoculum) (Fig. 4) the organism rapidly reaches maximum size and displays greatest pleomorphism at a time coinciding with the end of the lag period (Fig. 5). At this point the majority of the cells are binucleate and subsequently two large daughter cells are produced on division (Fig. 6). Progressive divisions result in a gradual decrease in cell size until at the end of the division cycle (10–12 hours) coccoid cells, identical in appearance with those of the original inoculum, are produced (Figs. 6–9).

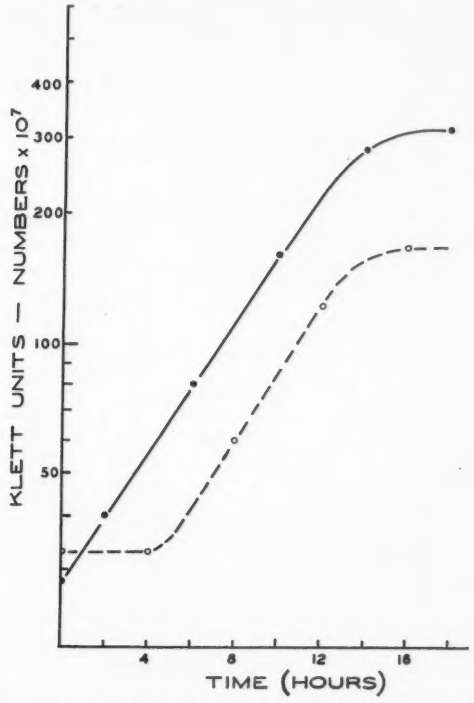

FIG. 2. Turbidity and numbers of *A. globiformis* growing in basal salts medium. Solid line, turbidity. Broken line, numbers of bacteria.

Comparable studies on the growth of *A. globiformis* in a basal salts medium (B) are presented in Fig. 2. Again, growth of the cells started immediately on inoculation and continued exponentially until the maximum stationary phase was reached. A lag period of 4 to 4.5 hours was again noted prior to cell division. Growth rates were considerably slower in medium B, with a generation time of 240 minutes as opposed to 82.5 minutes in medium YS. Microscopic examination of the developing organisms showed only a slight increase in cell size during the lag phase with a rapid reversion to the normal coccoid cells during logarithmic growth (Figs. 10–12).

Numerous reports have appeared in the literature on the extensive pleomorphism of *Arthrobacter* cultures in rich organic media (milk, sauerkraut juice, etc.). In the present studies a complex synthetic medium of Henderson and Snell (6) was selected as one sufficiently enriched to leave little to be desired from a nutritional standpoint. Growth of *A. globiformis* in this medium is presented in Fig. 3. The rate of growth was much enhanced, with the final cell mass being almost three times that found in medium YS. Considerable

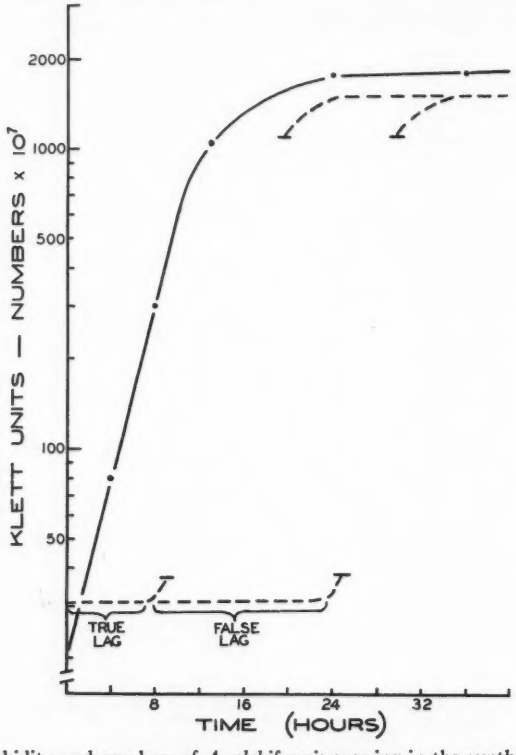

FIG. 3. Turbidity and numbers of *A. globiformis* growing in the synthetic medium of Henderson and Snell. Solid line, turbidity. Broken line, numbers of bacteria.

FIGS. 4–12. Photomicrographs (ca. 1450×) of *A. globiformis*. Fig. 4. Zero hours, medium YS. Fig. 5. Four hours, medium YS. Fig. 6. Six hours, medium YS. Fig. 7. Seven hours, medium YS. Fig. 8. Eight hours, medium YS. Fig. 9. Twelve hours, medium YS. Fig. 10. Zero hours, medium B. Fig. 11. Three hours, medium B. Fig. 12. Six hours, medium B.

FIGS. 13–21. Photomicrographs (ca. 1450×) of *A. globiformis* grown in the medium of Henderson and Snell. Fig. 13. Zero hours. Fig. 14. Three hours. Fig. 15. Five hours. Fig. 16. Six and one-half hours. Fig. 17. Eight hours. Fig. 18. Nine hours. Figs. 19–21. Twenty-four hours.

difficulty was encountered in determining cell numbers accurately in this medium. Under the growth conditions of media B and YS, binucleate cells were produced during the lag phase followed by normal fission. Growth in the enriched medium resulted in multinucleate cells which frequently failed to fragment to any degree until 24 hours or later. Sacks (10) has previously noted deceptively low counts of *A. citreus* due to failure of the cells to fragment. Repeated growth experiments under identical conditions failed to show any conformity as to time of fragmentation. As a result a false lag with respect to bacterial numbers was invariably obtained. Microscopic examination of preparations of these organisms indicates that a true lag exists until about the eighth hour during which time the organisms increase tremendously in size and exhibit extreme pleomorphism (Figs. 13–17). By this time multiple crosswalls are present (Fig. 18) and to all intents the cells are ready for division. Fragmentation or partial fragmentation may then occur at any time up to 24 or 36 hours. In instances where the cells fail to fragment for extended periods, chainlike growth is frequently noted. Figures 19–21 illustrate the typical appearance of three 24-hour cultures of *A. globiformis* in the medium of Henderson and Snell. Despite the irregularity in time of fragmentation, the organism does revert to the normal coccoid form at the end of the growth cycle.

Studies were also undertaken to determine the effect of increasing osmotic pressure on cell enlargement under the conditions of this medium. Consequently the Henderson and Snell medium was prepared with a final concentration of 0.3 *M* sucrose. No changes in the nature of cell growth were observed in the hypertonic medium nor did it have any effect on time of fragmentation of the cells.

Discussion

In the studies presented, the growth characteristics of *A. globiformis* have been determined in three media of different nutritional levels. In all media the cyclic nature of its growth was readily observed. During the lag period cells exhibited varying degrees of enlargement. These ranged from a 2-fold increase in size in a basal salts medium to an 8- to 10-fold increase in an enriched synthetic medium. Cells reached maximum size just prior to division and at this time the pleomorphic forms typical of the genus *Arthrobacter* were observed. Following the lag period, successive cell divisions occurred with a gradual decrease in cell size and reversion to the original coccoid form. In the richer medium there was a definite tendency for the cells to remain attached, resulting in chainlike growth in the later stages.

Contrary to numerous published descriptions of members of the genus *Arthrobacter* the growth behavior of *A. globiformis* appears reasonably straightforward. Many species of bacteria exhibit considerable cell enlargement during the predivision phase of growth and the degree of enlargement frequently depends on the nutritional level of the growth medium (15, 16). *Arthrobacter globiformis* and other members of the genus are unusual in that enlargement results in marked elongation and pleomorphism of the cells. These cells are not to be confused with the pleomorphic and megalomorphic forms exhibited by many bacteria under periods of stress.

No 'cystite' forms of the type described by Sguros (12) or by Chaplin (2) have been observed under the cultural conditions of these experiments. It is felt that the germinating cystites of Sguros are really a misinterpretation of the 'tailing-effect' so readily observed on stained preparations of *Arthrobacter* species and referred to earlier by Taylor and Lochhead (14) and Clark and Mitchell (4). The more plausible 'cystites' referred to by Conn and Dimmick (5) and Jensen (7) and ably photographed by Chaplin (2) need further investigation. The tendency for *Arthrobacter* species to gross pleomorphism in enriched cultural media, or under such adverse conditions as vitamin deficiencies (3) or the presence of antibiotics (13), suggests that the afore-mentioned cystites may well be artifacts or naturally occurring protoplasts.

Perhaps the most striking observation in these studies is the marked increase in cell mass during the predivision lag as compared with the normal coccoid cells. The degree of enlargement in all cases varied directly with the nutritional level of the medium. Kjeldgaard *et al.* (8) have pointed out the paradoxical situation in which the fewer the synthetic activities required during growth the larger the cell. Schaechter (11) on the other hand suggests that the growth rate cannot increase unless the protein-synthesizing system (RNA) first expands. Studies on the cytochemical changes of *A. globiformis* cells during the phase of cell enlargement are under way and will be reported in a later communication.

Acknowledgment

The author wishes to express his appreciation for the effective technical assistance of Miss Joan B. Watson.

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STUDIES OF PIGMENTATION BY PSEUDOMONAS INDIGOFERA[1]

E. C. Sommer, W. S. Silver, and L. C. Vining

Abstract

A pseudomonad which deposited a large quantity of a blue extracellular pigment on the top of the colony was isolated from soil and pond water. It was found to be identical both physiologically and morphologically with *Pseudomonas indigofera*. Good pigmentation was obtained in a synthetic medium containing methionine, glutamine, arginine, and aspartic acid. The addition of other amino acids reduced growth and/or pigmentation. Chemical properties as well as spectral data strongly suggest that the pigment is indigoidine.

Introduction

A *Pseudomonas* isolate was found to produce an insoluble blue pigment exhibiting a coppery metallic luster in reflected light. Since organisms of this type are comparatively rare and the isolate could not be positively identified from information available in the literature, a detailed study of both the organism and its pigment appeared necessary. As a result of this, the pigment was found to be very similar to, if not identical with, indigoidine, an insoluble blue pigment first described by Elazari-Volcani (2) in connection with *Pseudomonas indigofera*, and later found by Kuhn and Starr (3, 5) in *Corynebacterium insidiosum* and *Arthrobacter atrocyaneus*. Its chemical structure has not yet been reported.

Morphological and physiological characteristics of the microorganism strongly suggest its identity with *Pseudomonas indigofera*. Further characteristics of this species, which has only once previously been described in the literature (2), as well as an account of factors found to influence pigmentation in a simple defined medium, are reported below.

Materials and Methods

Cultures

The organism employed in this study, *Pseudomonas* sp., was isolated in 1957 from a plate discarded in a general bacteriology class laboratory in which samples of pond water and soil, from the University of Florida campus, had been plated.

Cultures of *Pseudomonas indigofera*, used for comparative purposes, were: No. 2368, from the collection of Dr. C. B. Van Niel, Pacific Grove (thought to be from Elazari-Volcani's original isolate); No. 2369, isolated at Pacific Grove by Dr. B. McFadden, Washington State University; and No. 2370, isolated from Delft canal mud by Dr. N. Walker, Rothamsted.

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Contribution from the Department of Bacteriology, University of Florida, Gainesville, Florida (E.C.S. and W.S.S.) and the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan (L.C.V.).

Taken in part from a Thesis submitted by E. C. Sommer to the Graduate Council of the University of Florida in partial fulfillment of the requirements for the degree of Master of Science.

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Media

The medium used for the large-scale production of the pigment contained, per liter, 40 g Trypticase Soy Agar (TSA), 5 g agar, 1 g yeast extract, 5 g glucose, and an excess of calcium carbonate added prior to autoclaving.

The basal medium used in the synthetic media study contained, per liter, 16 ml 0.2 *M* phosphate buffer (pH 7.0), 0.04 g $MgSO_4.7H_2O$, 0.002 g $MnSO_4.2H_2O$, 0.002 g $FeSO_4.7H_2O$, 2 g NaCl, 20 g agar, with the pH adjusted to 7.3–7.4.

Production and Isolation of the Pigment

Petri plates containing the production medium were heavily streaked with three or four lines of inoculum, ca. 1 cm wide, crossed by three or four more such lines perpendicular to the first set, from a 24-hour TSA slant. After 3 days' growth at 25° C, the cells and pigment were loosened from the agar surface with a bent glass rod, such as is used for streaking plates, and washed off with a minimum of distilled water. The suspension was centrifuged at low speed and the residual pigment washed repeatedly in the same manner until free of cells by microscopic examination. It was then dried under vacuum at room temperature and stored in a desiccator in the dark.

The Synthetic Media Tests

The various synthetic media were prepared in 50-ml amounts from which three plates were poured. The pH was adjusted prior to the addition of the agar and was rechecked after autoclaving. One-tenth of a milliliter of a cellular suspension (Klett reading of 80, filter No. 42, diluted to 10^{-5}) was spread on the surface of the various media to be tested.

The pigmentation was scored by visual estimation of color intensity viewed by transmitted light, a method which obviates errors due to different degrees of luster. The scoring of both growth and pigmentation was based on: 0, no growth or pigmentation; trace, a trace amount of growth or pigmentation; 1 through 4, an ascending gradation of pigmentation or growth with 4 being very good. In some cases, higher numbers were used when the pigmentation warranted it.

Results

The Organism

Morphology

Microscopic examination of the isolate revealed Gram-negative, slightly curved rods occurring singly and in pairs, the predominant arrangement being pairs. The cells, on an average, measured 2.4 μ in length and 0.7 μ in breadth. The isolate was actively motile in wet mount, and both flagella stain and electron microscopy revealed a single polar flagellum (Fig. 1).

Characteristic colonies appeared on Trypticase Soy Agar after 48 hours. The surface was smooth, the form circular, the elevation convex, and an entire margin was exhibited. Daughter colonies appeared at varying lengths of time after the parent colonies had reached their maximum size.

Physiology

The isolate was found to be a strict aerobe which oxidized glucose aerobically with the production of acid; the fermentative ability of the isolate was

PLATE I

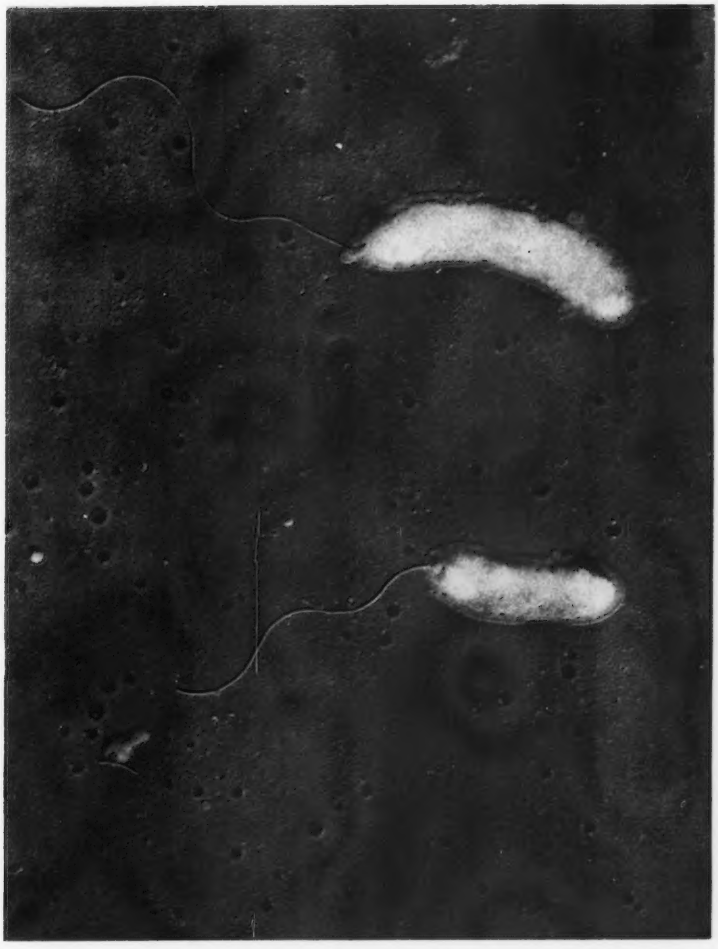


FIG. 1. Electron micrograph of pseudomonad showing polar flagellation. Phillips EM-100 Electron Microscope, platinum shadowed, ×23,000. (Courtesy Mr. T. Carlisle.)

negative on all sugars tested. In the presence of a suitable nitrogen source, ammonium sulphate, neither citrate nor acetate, was utilized as a sole carbon source for growth; either gelatin or succinate supported a trace amount of colorless growth; ethanol supported growth without pigmentation; glycerol or glucose supported both growth and pigmentation. The isolate was indole-positive, nitrite was produced from nitrate, and litmus milk became alkaline after a 7-day incubation period. The organism did not hydrolyze starch, casein, fat, or gelatin; lecithin was not attacked; hydrogen sulphide was not produced; nitrate was not reduced to free nitrogen nor was it converted beyond the nitrite reduction stage to ammonia.

Surface colonies of the isolate produced, in addition to the blue pigment, a yellow, water-soluble pigment. Subsurface colonies were pale yellow and lens-shaped.

Comparison with Pseudomonas indigofera

The characteristics of the isolate suggested its similarity to a group of pseudomonads designated by Sneath (4) as the "indigo" bacteria, which includes *Pseudomonas indigofera.* After an examination of the pigment had shown its similarity to indigoidine, isolates of this organism were obtained from other laboratories for comparison. This showed that all of the organisms tested were physiologically indistinguishable, but morphologically quite diverse, ranging from short, slightly curved or straight rods (strain No. 2368) to long, curved rods in chains (strain No. 2370). Strain No. 2368, the one thought to be from Volcani's original isolate, was both morphologically and physiologically identical with the isolate. An independent comparison in the laboratory of Dr. M. P. Starr yielded similar results.

Nutritional and Media Study

With the exception of a small amount formed at the liquid–glass–air interface, no pigment could be obtained when the organism was grown in liquid media. On semisolid complex media, pigmentation was found to be reduced by a crowding effect obtained in cases of confluent growth or on plates containing closely spaced colonies; the resultant growth was almost entirely translucent yellow with traces of blue at the periphery. The plating of a washed or diluted inoculum to obtain uncrowded growth conditions resulted in extreme variation in the pigmentation of individual colonies. Variation was diminished by transferring the pigmented cells from a slant directly to the production medium and the method finally chosen is described under the section Materials and Methods. A large increase in pigmentation was produced in an atmosphere obtained by burning a candle in a sealed desiccator. Under such conditions an inoculum of washed or diluted cells, giving plates of well-isolated colonies, resulted in good pigmentation. The influence of the gaseous atmosphere on the amount of pigment formed was also observed when plates were incubated in various mixtures of oxygen, nitrogen, and carbon dioxide. Pigmentation was heaviest with oxygen concentrations of 5 to 10% by volume at a carbon dioxide concentration of 1%.

Since the Sheffield peptone, N-Z Amine Type YT, was found to serve as an excellent substrate for pigment production, it seemed probable that a simple synthetic medium which would prove useful for future biosynthetic

work might be developed from known constituents. Testing of this supposition indicated that methionine was required for growth in all of the amino acid media devised, but would not support growth when employed as a sole source of carbon and nitrogen. The results of one experiment in which methionine was combined with various other amino acids are shown in Table I. Glutamine, which in combination with methionine (medium B) allowed both growth and pigmentation, was subsequently found to stimulate pigmentation in all of the synthetic media tested. Further supplementation with arginine increased both growth and pigmentation (medium C); the addition of either aspartic acid (medium D) or asparagine (medium E) to the methionine–glutamine–arginine medium doubled pigmentation but did not increase growth. The substitution of glutamic acid for aspartic acid or asparagine (medium F) increased pigmentation but to a lesser extent in the usual 3-day incubation period. However, upon longer incubation, the effect of the glutamic acid was greater than that of either the aspartic acid or the asparagine

TABLE I

Synthetic media for growth and pigmentation

Medium	Components*	Growth, 3-day	Pigmentation	
			3-day	4-day
A	Meth	0	0	0
B	Meth–Glu	2	1	1
C	Meth–Glu–Arg	3	2	2
D	Meth–Glu–Arg–Asp A	2	4	4
E	Meth–Glu–Arg–Asp	2	4	4
F	Meth–Glu–Arg–Glu A	2	3	5
Control	TSA	3	3	3

*L-Isomer per 10 ml medium: methionine (Meth) 4.8 mg, glutamine (Glu) 12.0 mg, arginine (Arg) 7.6 mg, aspartic acid (Asp A) 3.1 mg, asparagine (Asp) 6.2 mg, glutamic acid (Glu A) 11.3 mg. Added to salts' solution described in text.

TABLE II

Effect of individual amino acids on growth and pigmentation in a synthetic medium*

Amino acid added	Amount (mg per 10 ml medium)†	Results (3 days)	
		Growth	Pigmentation
Phenylalanine	10.6	3	Trace
Proline	41.2	4	Trace
Tryptophan	2.8	0	0
Tyrosine	7.2	3	2
Histidine	12.4	3	2
Lysine	30.0	3	3
Cystine	2.0	0	0
Alanine	13.2	3	2
Serine	25.6	3	3
Threonine	7.5	2	Trace
Leucine	19.5	0	0
Isoleucine	10.2	3	Trace
Glycine	6.8	0	0
Valine	11.4	0	0
Basal medium		3	3

*NOTE: The amino acid basal medium was medium D of Table I.
†The amount of L-isomer present.

and it was found to be required for the production of a luster comparable with that produced on complex media.

The effect of other individual amino acids on pigmentation was tested using the methionine–arginine–glutamine–aspartic acid medium as a basal medium. It was found that under the test conditions employed no single amino acid addition increased pigmentation; in fact, many inhibited pigmentation as well as growth (Table II). Thus the methionine–arginine–glutamine–aspartic acid medium was the best simple defined medium for good growth and pigmentation that was devised.

The Pigment

Morphological Observations on Pigmentation

The extracellular blue pigment which was present was observed both to cover and to underlie the cells, which themselves were light yellow. Upon removal of the cells, a blue ring, consisting of flakes of the pigment, remained in the agar. The colonies appeared dark blue to transmitted light; in reflected light, a coppery metallic luster was observed.

Microscopic observation of the pigment revealed that it was composed of flakes which assumed a regular shape and size. The diameter of the flakes was generally larger than the breadth of the cells and no definite connection could be observed between the cells and the flakes. The flakes appeared instantaneously, at first as irregular masses which, after some time, assumed regularity in size and shape. Phase contrast microscopy revealed the cells to be homogeneous in density both prior to and after pigmentation.

Chemical Nature of the Pigment

The pigment was insoluble in most common laboratory solvents, but dissolved to some extent in dimethylformamide, pyridine, and dimethyl sulphoxide, yielding stable blue solutions. Solutions in morpholine or ethylenediamine slowly decomposed, becoming finally yellow. The pigment was insoluble in aqueous ammonia, or sodium bicarbonate, but dissolved easily in sodium hydroxide to give a blue color which rapidly changed to yellow. Stable, aqueous solutions of the pigment could be obtained by suspension in a saturated solution of versene (50 ml) and addition with stirring of 1.0 *N* KOH (12 ml). As soon as all the pigment was dissolved, 0.2 *M* phosphate buffer (40 ml, pH 6.5) was added before the solution turned yellow. Several other chelating agents (α,α'-dipyridyl, citric acid, salicylaldoxime, potassium cyanide, sodium azide) could be used in place of versene. In concentrated sulphuric acid the pigment dissolved readily to an orange-yellow solution, from which, on careful dilution, a transient blue solution was obtained. Unstable blue solutions were also obtained with other aqueous mineral acids.

Samples of the pigment obtained as described under the section Materials and Methods, or by precipitation with ether or acid from clarified aqueous solutions prepared by the versene treatment, showed absorption maxima at 598 mμ in dimethylformamide or buffered aqueous versene and 602 mμ in pyridine.

Because of the close similarity of these properties to those reported for indigoidine (3, 5), samples of this substance isolated from *Pseudomonas indigofera* and *Arthrobacter atrocyaneus* were obtained for comparison. Figure

2 shows the infrared spectra of the three samples, and strongly suggests that they are identical. Supporting evidence comes from the visible spectra, which were also essentially identical, from the absence of melting point below 315° C in all samples, and from the similarity in elementary analyses. Acetyl derivatives prepared from the pigments of the Florida isolate and of *A. atrocyaneus* by treatment with pyridine and acetic anhydride were obtained as red needles (from nitrobenzene) not melting below 300° C. Their visible (λ_{max} 512 mμ in pyridine) and infrared absorption spectra (Fig. 3) were very similar.

A versene-stabilized aqueous solution of the pigment was reduced to a pale yellow color with sodium hydrosulphite, the blue color being regenerated by vigorous shaking in air. This could be repeated several times, although the blue color became progressively lighter on each reoxidation, and suggests the presence of a redox system such as exists in pyocyanine and other phenazine pigments commonly found in pseudomonads. Photoinstability, a property also common to phenazine derivatives, was also observed. A saturated solution of the pigment in dimethylformamide exposed to intense illumination from an incandescent lamp became yellow after 12 hours. However, distillation of the pigment with zinc dust failed to yield phenazine (1).

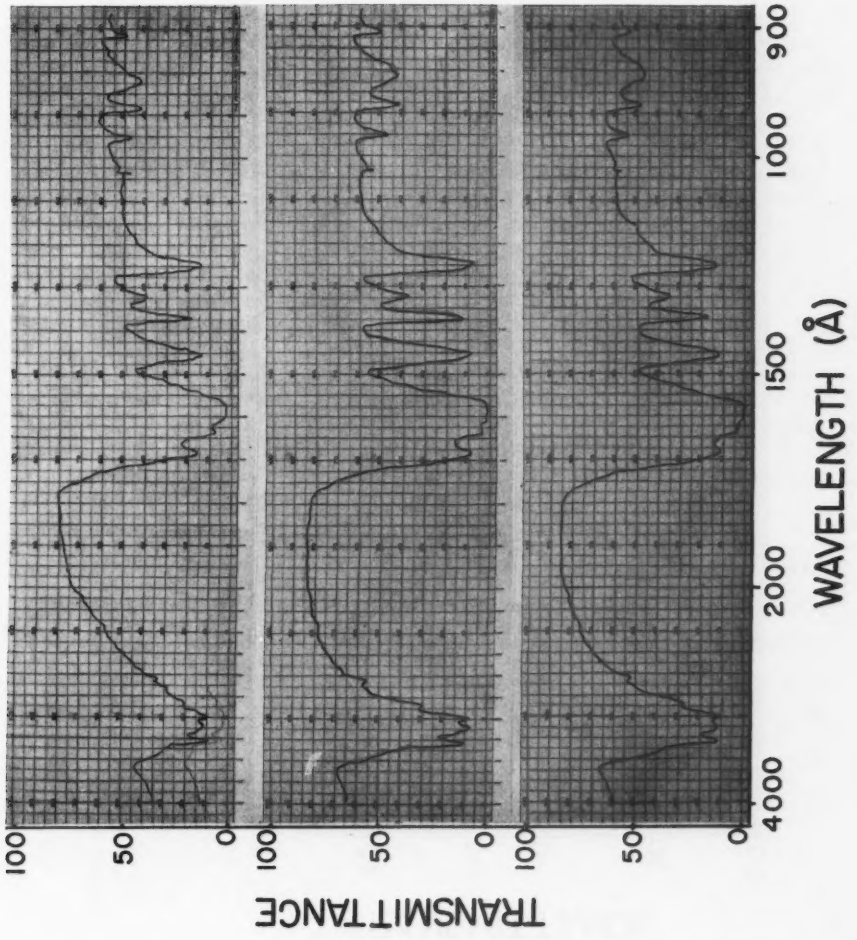
Consideration of the elementary analysis and spectra of the pigment suggests that it may be an aromatic nitrogen heterocyclic compound. The absence of a carbonyl absorption band which might be attributable to an ester in the infrared spectrum of the acetylated material indicates an N-acetyl derivative and corresponds with the loss of some absorption peaks in the NH stretching region compared with the parent compound. The failure to remove completely absorption in this region by acetylation may be due to the presence of inert NH groups, and, together with the presence of carbonyl absorption in the 1630–1685 cm^{-1} region, suggests a lactam structure of the type found in hydroxypteridine derivatives.

Discussion

It may be stated that the isolate described here is physiologically and morphologically indistinguishable from Volcani's *P. indigofera*. Factors which affected pigmentation—temperature (optimum = 25–28° C), pH (optimum = pH 7.1–7.3), "crowding", gaseous oxygen tension, and physical state of the medium—all influenced pigmentation in a manner similar to that reported by Volcani (2). Although Volcani's enrichment cultures were grown on citrate–nitrate broth, the strain described here did not grow on citrate as a sole carbon source on an ammonium-salts agar.

The composition of the medium had a profound effect on the production of the blue pigment as well as the diffusable yellow substance. It is noteworthy that, although pigmentation on complex media was very variable, pigmentation on synthetic media was almost always uniform, i.e., all colonies on synthetic media supporting pigmentation were pigmented. The usual variation encountered on complex media was physiological and not genetic

FIG. 2. Comparison of the infrared spectra of indigoidine and the pigment of the isolate. Upper curve, pigment of isolate; center, indigoidine from *Pseudomonas indigofera;* lower, indigoidine from *Arthrobacter atrocyaneus*. Perkin–Elmer Infrared Spectrophotometer, KBr disk.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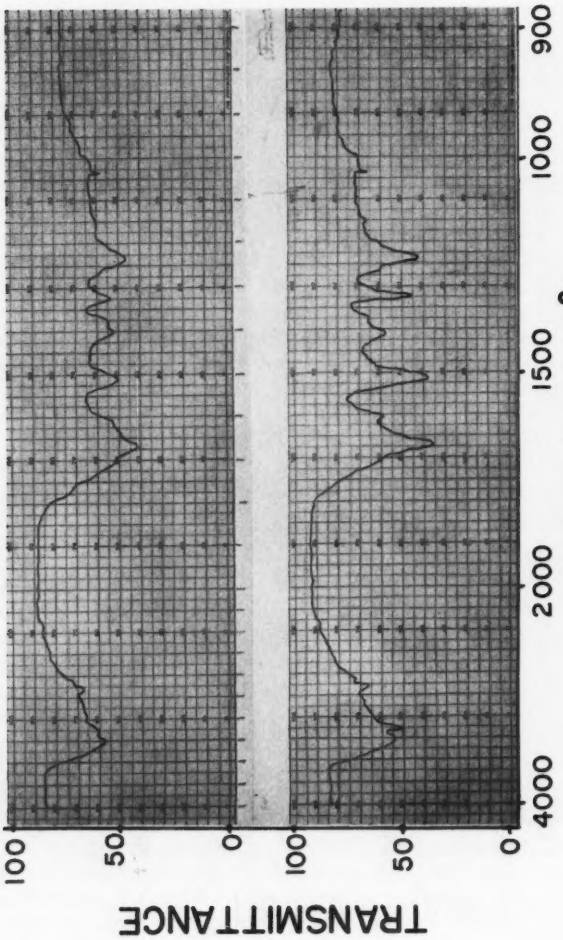

FIG. 3. Comparison of the infrared spectra of acetyl derivatives of indigoidine and pigment of the isolate. Upper curve, acetyl derivative of indigoidine from *Arthrobacter atrocyaneus*; lower, from pigment of isolate. Perkin–Elmer Infrared Spectrophotometer, KBr disk.

in nature since subcultures of nonpigmented, noncrowded colonies were pigmented upon transfer. A few, stable, nonpigmented variants were obtained during the course of the study but their incidence was infrequent.

The simplest synthetic medium devised which supported both good growth and pigmentation contained methionine, arginine, and glutamine or glutamic acid. Asparagine or aspartic acid were also effective stimulants of pigmentation. A variety of other amino acids, added to the simple synthetic medium, either had no effect on or reduced pigmentation and, in some cases, prevented growth. The great variation in the effectiveness of various peptones in stimulating pigmentation may thus be related to the amount of certain amino acids present.

The spectral data presented, as well as solubility and other properties of the pigment, although not necessarily providing irrefutable evidence, lend credence to the inferred identity of this pigment and indigoidine. The nature of the yellow, water-soluble substance as well as any possible relation to the blue pigment is not known. However, in every experiment performed, reduced blue pigmentation was invariably accompanied by an increased yellowing of the agar so it is possible that the production of the two substances is, in some unknown manner, linked.

Acknowledgment

Cultures of *Pseudomonas indigofera* and samples of indigoidine were kindly made available by Dr. M. P. Starr, Department of Bacteriology, University of California, Davis, California. The authors also wish to thank Dr. Starr for information on the results of independent comparisons of the organism and its pigment made in his laboratory.

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EXPERIMENTAL COXSACKIE B-3 INFECTION IN THE HIBERNATING SQUIRREL AND BAT[1]

GEORGE DEMPSTER, E. IRENE GRODUMS, AND W. A. SPENCER

Abstract

It has been shown for the first time that Coxsackie B-3 virus will infect hibernating mammals such as the ground squirrel and the bat, in both the hibernating and nonhibernating states. Pathological changes in the heart and brown fat of squirrels in hibernation were comparable with those observed in mice. In squirrels the brain appeared to be relatively resistant, although the region of the olfactory bulb was often involved. Although no lesions were observed in bats killed in hibernation, a few died with meningoencephalitis. Viraemia was observed in both species and found to be more persistent in the bat.

Biochemical studies were conducted upon the normal brown fat of the different species, and it was interesting to find that whereas the morphology and lipid content (total lipid and phospholipid) of the young mouse, adult squirrel, and adult bat were very similar, the corresponding characters of adult mouse brown fat showed marked differences. The significance of the resemblance of young mouse brown fat to that found in the hibernating mammal is being assessed further in the light of the function of the brown fat and its susceptibility to Coxsackie group B virus infection.

Introduction

The present paper is an extension of current studies (10) upon the role of lipids in the growth and pathogenesis of neurotropic viruses. The group B Coxsackie viruses were selected because of their peculiar tropism for the brown fat tissue, and the prototype B-3 virus exhibits this character in marked degree. Furthermore, these viruses produce marked damage, not only in this tissue, but also in the brain of the suckling mouse. In both these sites, active lipid metabolism is in progress at the time of high susceptibility.

Aronson *et al.* (1, 2), studying the effect of cortisone in experimental poliomyelitis, indicated that the brown fat might play an important role in the development of infection in the hamster, and since the present studies were commenced, there have been several reports concerning the significance of the role of brown fat in natural and experimental neurotropic disease.

Sulkin (18) demonstrated successfully that rabies virus inoculated intramuscularly into bats would locate in the brown fat tissue and even multiply in that site. Later Sulkin *et al.* (19) reported the isolation of St. Louis encephalitis and Japanese encephalitis virus from the brown fat of insectivorous bats inoculated subcutaneously at a site remote from the brown fat tissue. Bell *et al.* (3) have recorded isolations of rabies virus from the brown fat of naturally infected bats. Thus it would appear that an affinity for this tissue is common to several important groups of neurotropic viruses.

Biologists and others have speculated that the brown fat may play a very special role in the physiology of hibernation despite the fact that the same tissue occurs in relatively large amounts in nonhibernating species.

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Contribution from the Department of Bacteriology, Medical College, University of Saskatchewan, Saskatoon, Sask. Supported by a Federal Public Health Research Grant of Canada, and also aided by a grant from The National Foundation.

Of the hibernating species, the hamster has been shown to be susceptible to Coxsackie B infections but this animal only hibernates for short intervals. It was of interest, therefore, to find out whether Coxsackie B-3 infection could be established in a "deep" hibernator for it was felt that if this could be accomplished, much would be learned about the function of the brown fat, the pathogenesis of the virus infection, and the possibility of this particular tissue functioning as a reservoir for the overwintering of neurotropic viruses in general.

Materials and Methods

The golden-mantled ground squirrel (*Citellus lateralis*) and the big brown bat (*Eptesicus fuscus*) were used as experimental animals. The golden-mantled squirrel was specially selected on the advice of Dr. E. T. Pengelley of the Department of Zoology, University of Toronto, who considered it to be admirably suited for hibernation studies in the laboratory because of the length and depth of its hibernation periods. Earlier we had tested and rejected the species *Citellus richardsonii*, which, although readily available, was found to be a poor hibernator. The bats were included in these experiments because of their unique thermoregulatory mechanism; they have been claimed to hibernate whenever inactive (5, 7, 11, 13, 17). The species *Eptesicus fuscus* was selected as La Motte had already reported success in experiments with Japanese B virus (16), and it can be maintained readily under laboratory conditions (14).

The golden-mantled squirrels had been trapped in the Rocky Mountains of British Columbia during July and August, 1959. The bats for this experiment were made available by Dr. R. Connell, Department of Veterinary Sciences, University of Saskatchewan, and Dr. L. G. Saunders, Department of Biology, University of Saskatchewan, and had been caught in Saskatchewan and Alberta.

One group of the test animals was inoculated in early fall before the hibernating season; another in winter while in deep hibernation. During the nonhibernating period the animals were kept at an environmental temperature of $22 \pm 2°$ C. In winter they were placed in a room which was maintained at 2° C.

Virological and Histological Studies

The Coxsackie B-3 virus used for inoculation was mouse-adapted and the history of this strain has been given previously (10). The stock has an LD_{50} in newborn mice by the subcutaneous route of $10^{-7.43}$. A standard inoculum of 0.25 ml of a 1:100 dilution of mouse carcass suspension was used in all inoculations. All the experimental animals were inoculated by the subcutaneous route—over the interscapular region. After inoculation they were examined once a day and killed at various intervals.

The heart, brown fat, and brain of inoculated animals which had died or been killed were examined for macro- and micro-scopical lesions and for the presence of virus. Suspensions prepared from the blood and also from one-half of each of the selected organs were tested for the presence of virus by inoculating suckling mice. Titrations were made to ascertain the concentra-

tion of virus found in the brain, brown fat, heart, and blood of the animals examined at different stages of hibernation after inoculation. The LD_{50} was calculated according to Kärber's method (12). The identity of the isolated virus was confirmed by neutralization tests with a specific Coxsackie B-3 hamster antiserum.

Histological examinations were made from the other half of the organs used for virus isolation. The tissues were fixed in formol calcium or Bouin's fixatives (6), embedded in paraffin, and multiple sections of each organ were stained mostly with haematoxylin and eosin, van Kossa (6), and Luxol-Schiff's stains (15).

Biochemical Studies

Normal brown fat from the suckling and adult mouse and from the adult bat and golden-mantled squirrel was subjected to comparative study. The lipids were extracted by the method of Bloor (4), or by the method of Folch, Lees, and Sloane-Stanley (9). Aliquots were dried and weighed to determine total lipid. Lipid phosphorus was determined by the method of Fiske and Subbarow (8). For comparison, the lipid content of the white fat was also determined.

A detailed description of the lipids of brown fat of these species will be the subject of another publication.

Results

The preliminary experiments established that Coxsackie B-3 virus would infect *Citellus lateralis* during a state of nonhibernation. Six of the squirrels used for this experiment were about 6 weeks old and were unable to feed themselves on solid food yet; eight were adults. It can be seen in Table I that following subcutaneous inoculation of the nonhibernating animal, a high concentration of virus was obtained 4 days later, from both the brown fat and heart tissue in young as well as in the adult squirrels. At this time, there was no detectable viraemia. In animals examined 14 days after inoculation, virus was not detectable.

TABLE I

Golden-mantled squirrels inoculated in the nonhibernating state

Age	Days after inoculation	Virus titer* in different organs			Titer in blood
		Brown fat	Brain	Heart	
Young (a pool of 3)	4	5.58	<2.00	4.47	<1 in one squirrel
	14	—	—	—	—
Adult (a pool of 4)	4	5.75	<2.00	5.50	—
	14	—	—	—	—

* Reciprocal of log of LD_{50} in mice not older than 24 hours, inoculated subcutaneously.

It was of considerable interest, therefore, to follow up this investigation and determine the potentialities for active infection in the hibernating animal.

The findings are contained in Table II. In this table, the titers expressed are those for individual animals and since only two or three of them have been examined at each stage, one has to be careful in drawing final conclusions. It should be pointed out, however, that viraemia was detected 15 days after inoculation and, at the same time, virus was found in the heart tissue and later in the brain when it was first examined. The virus had reached the highest titer about 4 weeks after inoculation.

TABLE II

Golden-mantled squirrels inoculated during hibernation

Days after inoculation	Virus titer* in different organs			Titer* in blood
	Brown fat	Brain	Heart	
4	3.13	N.E.†	—	—
4	—	N.E.	—	—
4	2.63	N.E.	—	—
15	4.38	N.E.	3.00	4.00
15	3.75	N.E.	1.75	3.25
28	5.94	3.00	4.75	6.50
28	4.32	2.00	2.63	3.50
37	—	<2.00	—	—
37	—	3.00	4.69	—
50	—	—	—	—
50	<2.00	2.63	2.87	—

* Reciprocal of log of LD_{50} in mice not older than 24 hours, inoculated subcutaneously.
† N.E. = Not examined.

The findings with Coxsackie B-3 virus inoculated into the bat, *Eptesicus fuscus*, are equally interesting and remarkable, and are presented in Tables III and IV. Virus was readily recovered from the pooled blood, brown fat, and heart of bats kept at 20–22° C when killed and examined 4 days after inoculation. Virus was not found at this time in the pooled brains of these animals.

TABLE III

Bats inoculated and kept at 20–22° C

Days after inoculation	Virus recovery*			
	Brown fat	Brain	Heart	Blood
4	8/8	0/10	8/8	10/10

* Organs pooled from three bats before testing in suckling mice. The numerator indicates number of mice infected, denominator number inoculated.

In the *hibernating bat* (Table IV), it would appear that there might be some significance attached to the fact that the virus was easily recovered from the brown fat throughout the period of observation, i.e., up to a period of 140 days (about 5 months). Virus was detected less frequently in the heart and brain and only after a considerable time had elapsed after inoculation—some 3–4 months. This delay in appearance may be real or apparent

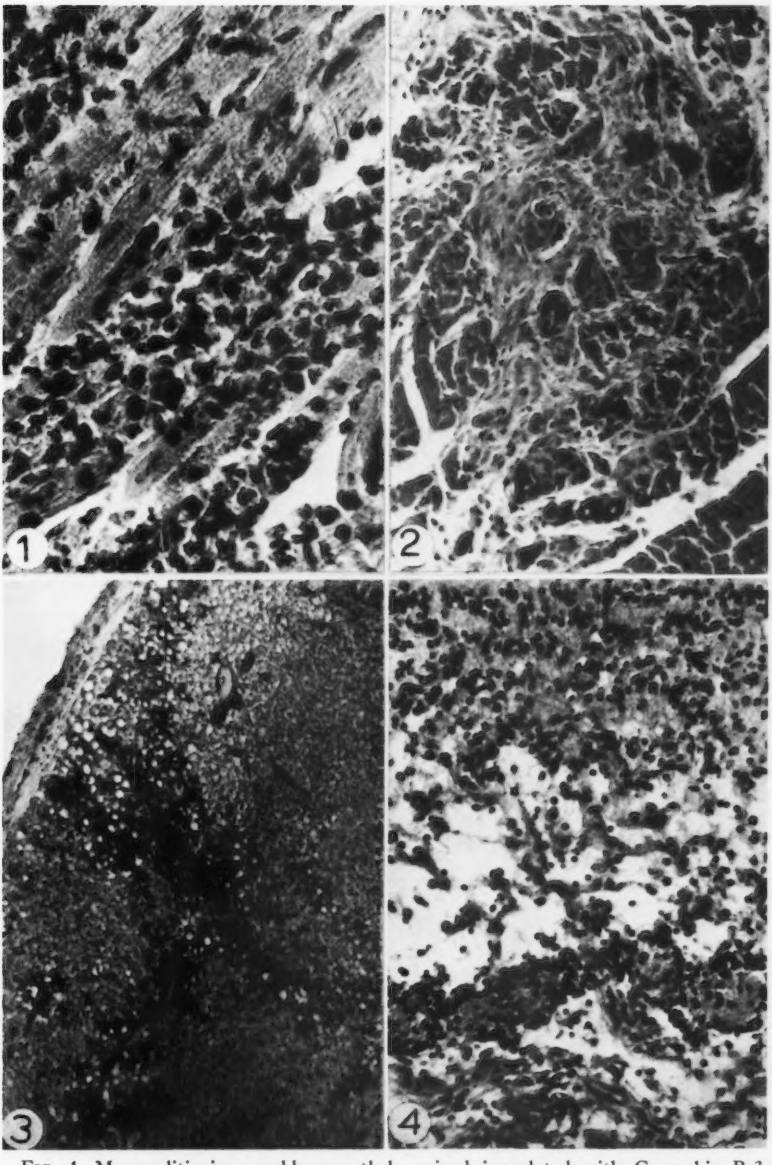

FIG. 1. Myocarditis in a golden-mantled squirrel inoculated with Coxsackie B-3, H.E. ×441. FIG. 2. Myocardial lesion thought to have resulted from a natural infection, H.E. ×184. FIG. 3. Brown fat pad of a squirrel inoculated and maintained in hibernation until killed 37 days later. Note the necrosis in the periphery of the lobules, Luxol-Schiff. ×64. FIG. 4. Pathological changes in the olfactory bulb of a golden-mantled squirrel infected with Coxsackie B-3 virus, Luxol-Schiff. ×184.

PLATE II

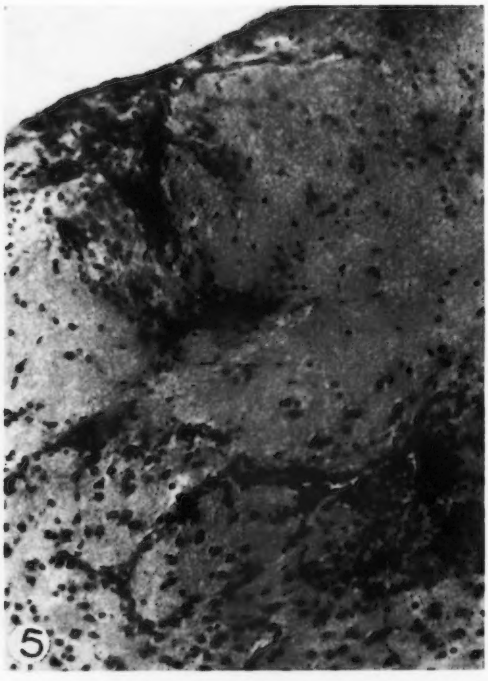

FIG. 5. Meningoencephalitis in a bat which was found dead 130 days after a subcutaneous inoculation with Coxsackie B-3, Luxol-Schiff. ×184.

and, while it is not possible to state from the numbers examined, the trend would suggest that it is probably real. There were indications too that an early strong viraemia may have developed into a less marked but very chronic condition displayed at 4 weeks and even at 4–5 months after inoculation.

TABLE IV

Bats inoculated and kept in hibernation at 2° C

Days after inoculation	Virus recovery*			
	Brown fat	Brain	Heart	Blood
4	8/8	N.E.†	N.E.	4/10
4	4/8	N.E.	N.E.	9/9
28	8/8	0/8	0/8	1/10
50‡	6/6	0/6	0/8	N.E.
78‡	8/8	0/8	N.E.	N.E.
92‡	8/8	2/8	N.E.	N.E.
130‡	10/10	4/10	9/9	N.E.
140	8/8	0/9	8/8	1/8
140	7/8	0/8	0/8	0/8

* The numerator indicates number of mice infected, denominator number of mice inoculated.
† N.E. = Not examined.
‡ The bat was found dead.

The Histological Findings

In the infected squirrel, irrespective of whether the animal was in the hibernating or the nonhibernating state, lesions were observed in the heart muscle and the lesions (Fig. 1) were identical with those observed in the Coxsackie-infected mouse. However, there was a certain difference since the lesions were not observed in the *hibernating* animal until 4–5 weeks after inoculation. A similar delay in the appearance of lesions in the brown fat was noted in the hibernating animal. Heart lesions not thought to be of Coxsackie origin were observed in three animals a few days after inoculation. These were thought to be natural infections of these wild animals due to other aetiological agents (Fig. 2).

The brown fat of the infected nonhibernating squirrel displayed a very mild inflammatory response which was completely absorbed 10 days later. In the hibernating squirrel, on the other hand, the reaction was more severe. It may be seen from Fig. 3 that the cells in the periphery of the brown fat lobules had undergone necrosis which is characteristic of Coxsackie B-3 virus infection, as observed in the suckling and young mice (10).

While lesions typical of Coxsackie B-3 infection were found with great regularity in the heart and brown fat, only on one occasion did the brain of the hibernating, inoculated squirrel show a typical virus encephalitis. However, in seven out of nine cases examined, there were pathological changes confined to the olfactory bulb (Fig. 4). The latter type of lesions were observed also in the young squirrels inoculated when not in hibernation.

Despite the fact that only a small number of bats were examined, the differences between the ground squirrel and the bat proved instructive. Lesions were not found in the heart of the hibernating bats, although virus was isolated upon several occasions. Degenerative changes were observed only occasionally in the brown fat and these were different in nature from the

pathological changes observed in either the mouse or the squirrel. The virus, however, was isolated without failure from the brown fat of every inoculated animal.

The fact that four of the inoculated hibernating bats died with evidence of meningitis and encephalitis and that in two of these animals Coxsackie B-3 virus was isolated from the brain is of interest but requires further study. It can, however, be said that the lesions (Fig. 5) did look similar to those observed in the suckling mouse. Pathological changes of the brain or meninges were not found in any of the infected animals which were killed for examination.

Biochemical Studies

Examination of the lipid content of mouse brown fat revealed a gradual accumulation of neutral lipid with age (Table V). Unlike mouse white adipose tissue, in which the glyceride content approaches the adult level within 2 weeks of birth, the mouse brown fat glyceride level rises sharply for three or four days after birth, and then increases slowly to adult level over a period of several weeks. During this period the relative amount of cytoplasm decreases, as reflected by the decreased level of phospholipid of the tissue.

Morphological studies conducted at the same time indicated that the observed changes in lipid content in the mouse brown fat parallel the morphological changes which occur as the animal ages.

TABLE V

Lipid content of brown and white fat*

Species	Age	White fat (total lipid)	Brown fat (total lipid)	Phospholipid (lipid P×25)
Mouse	4 days	38	29	3.5
	2 weeks	72	39	2.8
	10 weeks	80	59	1.9
Squirrel	Young	—	31	3.1
	Adult	88	30	3.5
Bat	Adult	82	34	3.4

* All values g/100 g fresh tissue. Phospholipid values are for grown fat.

Examination of the lipid content of the brown fat of the *hibernating* species used in these experiments reveals that values obtained for adults of these species are very similar to values obtained for the young mouse (Table V). The accumulation of neutral fat which occurs in mouse brown fat during aging was not observed in the hibernating species examined, and the morphological appearance of the brown fat of the adult hibernators is very similar to that of the brown fat of the young mouse.

Discussion

As a result of a logical extension of studies upon the effect of Coxsackie B-3 virus on the brown fat and brain of the mouse, it has been discovered that the brown fat of certain well-known hibernating mammals will support the growth of this virus. The golden-mantled squirrel, *Citellus lateralis*, is

a typical example of an animal which hibernates deeply during the winter months, and this species proved to be particularly susceptible to infection and even the adults developed virus titers in the brown fat and heart comparable with those found in the suckling mouse. It was noticeable that in those squirrels infected during hibernation, high virus titers were found in the brown fat and heart 2 weeks after inoculation. Lesions, however, were not detected until a period of 4–5 weeks after inoculation.

Any discussion of the present findings must take into account the possibility of naturally occurring infections of many different kinds in these wild animals. Lesions, for instance, were observed in the brain and heart of uninoculated animals and heart lesions attributed to a residual infection were seen in three of the inoculated squirrels. Despite this, the lesions described in the majority of the Coxsackie-infected animals bear striking resemblance to those found in the standard laboratory animal, the mouse. Moreover, virus was recovered and identified by specific neutralization tests from the squirrels showing typical Coxsackie B-3 lesions.

The bats differ from the true hibernators, or heterotherms, in that when awake and inactive, not only in winter but also in the summer months, their body temperature falls to that of the environment and only becomes raised when they are active as in flight. Hock (11), on account of this difference, has placed bats in a different metabolic classification. Inclusion of these mammals in the present studies has brought to light interesting and provocative differences in the virus–host relationship. In the bat that had been kept inactive continuously at 2° C, the virus had multiplied and caused viraemia. The occurrence of chronic viraemia and the persistence of the virus in the brown fat of the hibernating bat infected with Coxsackie B-3 virus may have wider implications with respect to the possible overwintering of enteric or neurotropic viruses.

In considering the age factor in this experimental infection, one should remember that the brown fat in the newborn and weanling mouse is peculiarly susceptible to Coxsackie B-3 virus, but as the animal ages, this susceptibility alters. In like manner, the morphological appearance and the lipid content of normal brown fat alters as the mouse grows up. This does not seem to be the case in the hibernating species examined and since the hibernators have retained their susceptibility in respect of this tissue, despite the aging process, one is led to wonder whether there is a more direct connection between these observations.

Discussion of these points cannot be adequately dealt with until further observations have been made with more animals at different time intervals and until information is available about the development of antibodies in these infected animals. Suffice it to say that the observed differences have prompted the authors to make a thorough study of the pathogenesis of the infection in the hibernating mammal, paying particular attention to the body temperature changes in the animal during hibernation.

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THE ROLE OF FISH AS CONVEYORS OF MICROORGANISMS IN AQUATIC ENVIRONMENTS[1]

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Abstract

Microbial analysis of 60 fish caught in three areas of Flathead Lake, Montana, substantiated the theory that fish carry a significant population of bacteria and fungi on their body surfaces and in their digestive tracts. Bacterial counts often were in the hundred thousands per square centimeter. Numbers and kinds of bacteria varied from fish to fish, showing no relation to location or kind of fish. Gram-negative, chromogenic rods were found most frequently. The ease with which these organisms were removed indicates that fish are important as passive carriers of bacteria in the aquatic environment.

There was no evidence that the fungi isolated were commensal with the fish or parasitic on them. Since all the fungus species isolated except one have been found in water or bottom mud of Flathead Lake, it would appear that fish operate also as passive conveyors of fungi. The idea that fish play a major role in microbial distribution is given substance by the fact that 48 species of fungi have been isolated from surface samples and 8 additional species from the contents of stomach and oesophagus.

Introduction

Concomitant with studies of the microbial population of Flathead Lake, Montana, by standard qualitative and quantitative procedures (12, 13) some of the means of distribution and persistence of this population have been investigated. One approach concerned the determination of the role that endemic fish may play as sources and distributors of microorganisms.

Whether a microbial population of a given body of water is endemic or introduced, the microorganisms constituting this population undoubtedly are subject to distribution by currents and also may be distributed by animals serving as active or passive carriers. It is known that the largest populations of bacteria are associated with solid surfaces, such as the sea bottom and bodies of marine animals (5). Fish, therefore, provide a means of investigating the role of animals as conveyors of microorganisms in a given environment. Numerous studies of bacterial populations derived from the surfaces of fish and their digestive tracts have been made, particularly for marine fish. These investigations have been summarized recently by Tarr (15) and Liston (7, 8). No comparable data are known for the isolation of fungi from fish, although Liston (8) noted molds and yeasts as 4% of his isolates from skate and lemon sole.

Materials and Methods

Samples from the lateral surfaces of 60 fish taken in Flathead Lake were obtained during the summers of 1954, 1955, and 1956. These fish represented 10 species of 7 genera (Table V). They were selected at random from fish caught in nets set for experimental purposes of the Fisheries Laboratory of the

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Montana Biological Station. Fish from 11 net sets were sampled (Table III). All of the fish were from three regions of the lake used previously in microbial sampling (12, 13).

Fish were swabbed just as the nets were being lifted from the water to reduce contamination to a minimum. A piece of sterile aluminum foil with an opening of 1 sq. cm was laid on the surface of the fish. The exposed opening was scrubbed three times with a sterile swab moistened in sterile water. The direction of swabbing was reversed each time. Both sterile cotton and sterile alginate swabs were used. Each swab was placed in a sterile water blank and stored in a refrigerator bag cooled to approximately 5° C. Plating was done immediately upon return to the laboratory. The cotton swabs were shaken vigorously. An equal volume of 10% Calgon solution was added to the alginate swabs before shaking. Analysis of gut contents was made by quantitative dilution and plating as soon as possible.

Dilutions for plating ranged from 10^{-1} to 10^{-4}. Sodium caseinate agar was employed as a standard, selective medium (3) although a variety of media were tried. Additional agars included fish extract agar, *Arthrobacter* medium, a casamino acids medium, and thiotone medium (11); Pringsheim's *Euglena* medium modified according to Funk (4). Of five media used for fungus isolations, rose bengal was the most satisfactory (10).

Plates, usually in replicates of three or five, were held at room temperature, approximately 20–25° C, for 3 weeks, then counted. Individual fungi were transferred to appropriate media for identification. After counting, bacterial smears were prepared. Selection was random until a total of 60 smears per fish had been prepared. Smears were stained by the Gram stain method.

Results

The bacterial populations per square centimeter of the lateral surfaces of the 59 fish from which bacterial counts were obtained are analyzed in Table I. In all, 76 platings were made of these 59 swabs. The populations were large but the number and kinds of bacteria per square centimeter could not be related to species of fish or to location of the net set (Tables I, II). Results were comparable for cotton and alginate swabs with either water or 5% Calgon as the diluent. Larger populations and a greater diversity of bacteria usually were isolated with sodium caseinate agar than with other media. Fish agar supported large numbers of bacterial colonies but sometimes these represented fewer kinds of bacteria. Counts are recorded as a range of the numbers occurring on all swabs from each location on a given agar.

Not only were the total numbers of bacteria larger than those observed previously in the water of Flathead Lake at the same locations (11, 12), but the percentages of chromogens were higher. Yellow and orange colonies were predominant; pink and red chromogens occurred in lower frequencies.

Bacterial morphology as determined for many of the swabs is recorded in Table I. Gram-negative rods were predominant; many were pleomorphic. Only one swab of those analyzed morphologically had a low percentage, 5%, of Gram-negative rods. A few Gram-positive rods (usually pleomorphic) and Gram-positive and Gram-negative cocci were found. None of these was per-

TABLE I

Bacterial populations on fish

Fish species and location of net set	No. of swabs	Medium used	Range or total no. of bacteria per sq. cm × 10^3	Percentage morphological types*					
				Chromogens	Gram − rods	Gram + rods	Gram − cocci	Gram + cocci	Yeasts
Catostomus catostomus (Forster)									
PB†	2	Na cas.	6.1–17.4	9.0–90.0	91.0	0	8.0	0	1.0
PB	1	Fish	12.1	7.0	—	—	—	—	—
BA†	1	Na cas.	102.0	76.0	93.0	5.0	1.0	1.0	0
Catostomus macrocheilus (Girard)									
PB	4	Na cas.	2.0–16.0	23.0–82.0	90.0–98.0	0–1.0	0	0–1.0	0.10.0
PB	1	Fish	18.0	18.0	—	—	—	—	—
YB†	1	Na cas.	21.5	89.0	96.0	3.0	0	1.0	0
Coregonus clupeaformis (Mitchell)									
PB	4	Na cas.	0.97–53.0	35.0–89.0	97.0–100.0	0	0–3.0	0–1.0	0
BA	1	Na cas.	330.0	79.0	99.0	0	0	0	1.0
BA	1	*Arthrob.*	215.0	82.0	100.0	0	0	0	0
YB	15	Na cas.	0.73–425.0	5.8–93.0	80.0–100.0	0–5.0	0–3.0	0–3.0	0–20.0
YB	3	Fish	2.9–49.0	2.0–55.0	93.0	0	5.0	2.0	0
YB	1	Casamino	14.3	89.0	94.0	1.0	5.0	0	0
YB	1	*Euglena*	6.5	85.0	99.0	1.0	0	0	0
Mylocheilus caurinum (Richardson)									
PB	3	Na cas.	3.5–21.9	54.0–83.0	99.0–100.0	0–1.0	0	0	0–1.0
BA	2	Na cas.	7.4–257.0	53.0–83.0	93.0–95.0	1.0–6.0	0	1.0	0–3.0
BA	1	*Arthrob.*	6.0	63.0	84.0	15.0	0	1.0	0
YB	1	Na cas.	0.31	54.0	5.0	0	3.0	3.0	89.0
YB	1	*Arthrob.*	0.27	33.0	—	—	—	—	—
Oncorhynchus nerka (Walbaum)									
PB	1	Fish	21.5	11.0	—	—	—	—	—

TABLE I (*Concluded*)

Fish species and location of net set	No. of swabs	Medium used	Range or total no. of bacteria per sq. cm × 10^3	Percentage morphological types*					
				Chromogens	Gram − rods	Gram + rods	Gram − cocci	Gram + cocci	Yeasts
Perca flavescens (Mitchell)									
PB	4	Na cas.	3.3–59.0	73.0–89.0	86.0–96.0	0	0–1.0	0–3.0	3.0–10.0
BA	1	Na cas.	2.7	9.0	—	—	—	—	—
BA	1	Fish	8.5	74.0	—	—	—	—	—
Ptychocheilus oregonense (Richardson)									
PB	3	Na cas.	1.7–113.0	64.0–91.0	93.0–100.0	0–3.0	0–1.0	0–3.0	0–1.0
BA	2	Na cas.	1.8–20.6	58.0–74.0	78.0–96.0	3.0–16.0	0	1.0–6.0	0
BA	1	*Arthrob.*	2.66	72.0	76.0	13.0	0	10.0	1.0
YB	2	Na cas.	0.69–48.9	4.0–33.0	71.0	11.0	0	0	18.0
YB	1	Fish	44.3	4.0	—	—	—	—	—
YB	1	Thiotone	0.45	30.0	91.0	1.0	0	0	8.0
Salvelinus alpinus malma (Walbaum)									
PB	3	Na cas.	8.7–135.0	85.0–90.0	92.0–99.0	0	0–8.0	0–1.0	0–3.0
BA	2	Na cas.	10.2–72.0	42.0–76.0	83.0–89.0	3.0–6.0	0	3.0–5.0	3.0–8.0
BA	1	*Arthrob.*	6.9	48.0	79.0	8.0	0	10.0	3.0
YB	5	Na cas.	0.18–289.0	46.0–86.0	82.0–100.0	0–5.0	0–6.0	0–5.0	0–8.0
YB	2	Fish	4.1–34.3	0.8–66.0	99.0	0	1.0	0	0
YB	1	*Arthrob.*	0.38	22.0	85.0	1.0	0	8.0	6.0
Salvelinus namaycush (Walbaum)									
PB	1	Fish	32.0	66.0	96.0	0	3.0	1.0	0

*Expressed as range of percentages when more than one sample is included.
†PB, Polson Bay; BA, Big Arm Bay; YB, Yellow Bay.

sistently present. Occasionally more than a few were found, notably on *Ptychocheilus oregonense*. Yeasts were observed fairly frequently, often in relatively large percentages.

TABLE II

Characteristics of the bacterial populations of fish

Total no. per sq. cm	No. of fish	% of chromogens	No. of fish
0–10	0	0–10	6
10–100	0	11–20	0
100–1,000	6	21–30	1
1,000–10,000	20	31–40	4
10,000–100,000	26	41–50	2
Above 100,000	8	51–60	7
		61–70	6
		71–80	13
		81–90	19
		91–100	2

Table II summarizes the data on the number of bacteria and the percentages of chromogens obtained. The majority of fish had between 1000 and 100,000 bacteria per sq. cm. Over 75% of the fish had more than 50% chromogens.

Plate counts for fungi by net sets and localities are listed in Table III. More revelatory than the plate count is the total number of fungus species isolated from each net set and the species recovered (Tables III; IV).

A total of 37 genera of fungi were identified from these plate isolations. Forty-eight species were found among them. All are Fungi Imperfecti and none is pathogenic to fish. Only five genera were represented by more than one

TABLE III

Fungus populations carried by fish in Flathead Lake

Date of sample	Location of net set	No. of fish	Kinds of fish	Range of counts, fungi per sq. cm $\times 10^3$	No. of fungus species	No. of fungus genera
7-9-54	YB	2	2	0.25	2	2
7-12-54	YB	5	5	0.25–17.5	11	9
7-5-55	YB	4	3	0.25–5.0	11	8
7-6-56	YB	10	6	0.10–0.33	20	18
7-10-56	YB	9	2	0.10–3.0	18	15
Totals:		30	18		40	32
7-16-55	PB	7	6	0.10–1.0	18	17
7-16-55	PB	1	1	1.50	4	4
7-12-56	PB	8	5	0.10–1.50	8	8
7-12-56	PB	6	5	0.16–0.20	6	5
Totals:		22	17		25	22
7-18-56	BA	5	4	0.16–0.20	10	10
7-18-56	BA	3	2	1.0	9	9
Totals:		8	6		13	13
Grand totals:		60	10		48*	37*

*From Table IV.

TABLE IV

Species of fungi carried by fish of Flathead Lake: their occurrence by species of fish and location; numbers of isolations by fish and net sets

Fungi	Fish species*										Location	Total no. of isolations	
	1	2	3	4	5	6	7	8	9	10		By fish	By sets
Alternaria tenuis			+					+	+		YB, BA	3	3
Aspergillus niger									+		BA	1	1
Aspergillus sp.		+							+		YB, PB	2	2
Aspergillus terreus			+				+				YB, PB	2	2
Basidiomycete								+			YB	1	1
Beauveria sp.			+					+			BA	2	2
Candida albicans			+		+			+			YB	3	2
Cephalosporium acremonium								+			YB, PB	2	2
Cephalosporium aspermum		+	+		+				+		YB, PB	8	1
Cephalosporium sp.		+	+						+		YB, PB	4	3
Cladosporium cladosporides					+						YB	1	1
Cladosporium elatum		+	+	+				+	+		YB, PB	7	4
Cladosporium herbarum	+	+	+					+	+	+	YB, PB, BA	22	6
Cladosporium macrocarpum			+		+		+	+		+	YB, PB	16	5
Coniosporium paradoxacum			+								YB, PB	2	2
Coniothyrina sp.					+				+		YB, BA	3	3
Fumago sp.								+	+		YB	2	1
Fusarium sp.	+	+	+					+	+		YB, PB	10	4
Geotrichum candidum		+	+					+	+		YB, PB	5	3
Gliomastix convoluta							+				YB	1	1
Hormodendrum hordei		+	+					+	+		YB, PB	3	3
Isaria sp.			+								PB	2	2
Itersonilia sp.							+				PB	1	1
Macrosporium sarcinaeforme			+						+	+	YB, PB	4	4
Monilia acremonium		+	+		+		+	+	+		YB, PB	14	5
Mucor sp.			+								PB	1	1
Mycelia sterilia		+	+		+		+	+	+		YB, PB, BA	11	3
Oidium tenellum	+										YB	1	1
Oospora variabilis			+		+			+	+		BA	4	2
Penicillium citrinum			+								YB	8	3
Penicillium commune			+						+		YB, BA	3	2

TABLE IV—(*Concluded*)

Fungi	Fish species*										Location	Total no. of isolations	
	1	2	3	4	5	6	7	8	9	10		By fish	By sets
Penicillium cyaneo-fulvum			+	+							YB	2	2
Penicillium egypticum					+						YB	1	1
Phoma hibernica	+	+	+		+		+	+	+		YB, PB, BA	20	6
Phomopsis sp.		+									PB	1	1
Pullularia pullulans			+				+		+		YB, PB	4	4
Pyrenochaeta sp.				+							YB	1	1
Pythium sp.				+							YB	1	1
Rhodotorula sp.					+		+	+	+		YB, PB, BA	9	4
Saccharomyces sp.			+				+	+	+		YB, PB, BA	8	5
Sporotrichum olivaceum		+									YB	1	1
Stilbella sp.	+		+								YB, PB	2	2
Stysanus stemonitis		+	+				+	+	+		PB, BA	6	3
Synnematium sp.			+								YB	1	1
Trichoderma album			+		+						YB, PB, BA	4	3
Trichoderma viride		+							+		YB	2	2
Verticillium candelabrum		+									YB	1	1
Volutella sp.					+						YB	1	1

*1, *Catostomus catostomus* (Forster); 2, *Catostomus macrocheilus* (Girard); 3, *Coregonus clupeaformus* (Mitchell); 4, *Coregonus williamsoni* (Girard); 5, *Mylocheilus caurinum* (Richardson); 6, *Onchorhynchus nerka* (Walbaum); 7, *Perca flavescens* (Mitchell); 8, *Ptychocheilus oregonense* (Richardson); 9, *Salvelinus alpinus malma* (Walbaum); 10, *Salvelinus namaycush* (Walbaum).

species. No fungus occurred on fish from all net sets. Some species were isolated from as many as 20 and 22 fish (Table IV). Seventeen species occurred only in plates from a single net set. Eight species were not recorded from either bottom mud or water samples in the same years, but had been recorded at least once during the period 1951 through 1953 (1). Only one species from the swabs has been found on fish alone, *Macrosporium sarcinaeforme*. There seems to be no reason to accord this with any significance other than chance and random sampling, since subsequently it has been found elsewhere (1).

Of the 48 fungus species isolated from the fish in these net sets, just 6 occurred on fish taken from all three areas; 18 were common to fish of two areas; and 24 occurred in a single area. The six species isolated from fish in all locations were: *Cladosporium herbarum*; a nonsporulating, white species, *Mycelia sterilia*; *Phoma hibernica*; *Rhodotorula* sp.; *Saccharomyces* sp.; and *Trichoderma album*. Of these *C. herbarum* and the sterile fungus were isolated from both water and mud as well as from fish during the sampling years, 1954–1956 (1). *Phoma hibernica* also occurred in the water of all three localities. No species was found to be common only to bottom mud of the three areas and to fish during the sampling period. Sixteen of the species isolated from fish are known from bottom mud and water of the lake during the sampling period (1), twelve of which occurred in the same year that they were isolated from the fish sample.

Table III records the occurrence and kinds of fish and fungi in relation to the individual net sets. Analysis of these data shows that the number of fungi ranged from 1 to 22 per fish (Table IV). The highest number of fungus species per swab was nine (Table V). Two swabs were negative although other swabs from the same kinds of fish had from two to eight species per fish.

In 1955 stomach and oesophagus contents of 12 fish were plated. Plate

TABLE V

Numbers of fungus species isolated from 10 species of fish

Fish species	Total no. swabbed	No. of fungus species/fish	Total no. of fungus species
Catostomus catostomus (Forster)	3	3 0 3	6
Catostomus macrocheilus (Girard)	5	6 5 1 3 5	15
Coregonus clupeaformis (Mitchell)	20	1 5 4 6 7 2 4 2 6 7 5 4 4 3 3 5 1 1 2 2	30
Coregonus williamsoni (Girard)	1	2	2
Mylocheilus caurinum (Richardson)	6	1 3 3 2 1 9	14
Onchorhynchus nerka (Walbaum)	1	1	1
Perca flavescens (Mitchell)	5	4 4 4 2 4	11
Ptychocheilus oregonense (Richardson)	7	3 2 7 2 4 8 0	20
Salvelinus alpinus malma (Walbaum)	11	1 4 4 5 5 5 4 1 4 3 4	22
Salvelinus namaycush (Walbaum)	1	3	3

counts were similar to those of surface swabs with numbers ranging from 150 to 15,000 per ml. From these isolations a number of different fungi were obtained. In all, 21 species were identified, 8 of which were not found on the lateral surfaces. These species were: *Botrytis cinerea*, *Dactylium* sp., *Diplocladium* sp., *Hormiscium* sp., *Phialophora* sp., *and Spicaria simplicissima*, all Fungi Imperfecti; and two Ascomycetes: *Chaetomium* sp. and *Emericellopsis humicola*. One fungus, *Dactylium* sp., is unique for Flathead Lake records through 1960 (1).

The data on bacterial populations in the gut were too limited to be considered significant.

Discussion

The microorganisms isolated should be representative of the microbial populations carried by the fish since the fish were subjected to a minimum of handling. Neither the size of the bacterial population, nor the kinds of bacteria present on the surface of the fish sampled, showed correlation with the locality or kind of fish. The number of bacteria per square centimeter of fish surface was high in comparison with the number per milliliter of water in Flathead Lake (11, 12). Considerable variation in numbers per square centimeter was observed for different fish and for the same kind of fish in a given net set. Liston (8) reported seasonal variation in counts on marine fish but since all the fish sampled in this work were taken in the same month, no data on seasonal variation were obtained. Similar variation in the kinds of bacteria present was noted among representatives of the same species of fish in one net set, as well as among different kinds of fish in the same net set or in different net sets. Liston (8) noted a distinct relationship between species of bacteria and species of fish. This study did not include the determination of bacterial species. Cultures were distinguished on the basis of chromogenesis, Gram reaction, and general morphology. Using these criteria there did not appear to be specific bacteria associated with specific fish.

Chromogenesis was common among the bacteria on these fish. Gram-negative rods, the predominant form observed, are usually the most numerous organisms in both the water and mud of Flathead Lake (11, 12, 13). The presence of various sizes of monomorphic Gram-negative rods as well as pleomorphic Gram-negatives indicated that many species and possibly many genera of bacteria were present. Some Gram-positive rods were usually present along with both Gram-positive and -negative cocci. On fish of Flathead Lake the chromogenic, Gram-negative rod is the most common bacterial type.

Some of these bacteria could be indigenous in the sense proposed by Liston (8). On the basis of the data reported here there is no evidence that a specific indigenous bacterial flora is associated with a particular kind of fish. Instead the fish flora appeared similar for various kinds of fish in the same environment.

There is no clear correlation between kinds of fungi and locality of the net sets, nor between numbers of fungus species isolated and the kinds of fish conveying them (Table IV). Only 7 of the 48 fungi isolated from surface swabs of fish occurred on 5 or more of the 10 fish species. Arranging the occurrence of the numbers of fungus species per fish species in classes from one to four, using intervals of 0.99, the class most commonly represented is 2.0 to 2.99. This

class had seven members. Two representatives fall below that class and one exceeds it.

The relationship that Liston (8) found between bacterial species and species of fish was not evident between fungi and fish. Because of repeated isolation of the same type of organism from the skin and gills of the two species of fish sampled, Liston (8) characterized the bacteria as commensal with the fish. If the fungus population of the fish in Flathead Lake is to be regarded as commensal in this sense, it should be possible to isolate the same kinds of fungi from the same kind of fish repeatedly. Using *Macrosporium sarcinaeforme* as a test case since it has occurred only on fish, the records indicate: 1 isolation from *Salvelinus namaycush* for which a single fish was swabbed; 2 isolations out of 11 swabs on *S. alpinus malma*; and 1 out of 20 fish representing *Coregonus clupeaformis*. Another fungus picked at random, *Alternaria tenuis*, also appeared in 1 of 20 swabs on *C. clupeaformis*; it occurred in 1 of 11 swabs from *S. alpinus malma* and 1 out of 7 swabs on *Ptychocheilus oregonense*. *Phoma hibernica*, the fungus of penultimate frequency, occurred on 7 of 10 fish species. Isolations per fish species were variable showing occurrence on 1 out of 3, 1 out of 5, 3 out of 20, 4 out of 6, 2 out of 5, 3 out of 7, and 6 out of 11 fish. Such data do not seem to support commensalism on a repeated isolation basis.

The bacteria of the fish gut have been regarded as a function of the food ingested since intestinal tracts of nonfeeding fish are virtually sterile (9). That the food then provides the population is obvious, although there may be modifying factors. The probability of an indigenous flora in the digestive tract has been questioned, but Liston (8) found evidence for it. The data on fungi isolated from the digestive tracts of fish in 1955 were too limited to provide any information about an indigenous fungus flora. If such should exist it would be comprised of species able to survive ingestion. Many fungi become nonviable following intestinal passage (6). Many other fungi, including Ascomycetes, are known to have a tolerance for such passage; some even require it (6, 16). It is, therefore, of interest that the only two species of Ascomycetes isolated from fish came from intestinal samples.

The total number of fungi isolated from all kinds of fish samples was 56. This represents approximately 31% of the total number of fungus species isolated from the water and bottom mud of Flathead Lake during the summers of 1951 through 1956 (1).

It is apparent from the data presented that the fish sampled carried a large and varied population of microorganisms. The counts should represent the typical condition as contamination was probably minimal and none of the fish observed or sampled appeared to be diseased. The numbers and kinds of microorganisms reported should be an indication of the flora carried by healthy, typical fish.

If the microorganisms are present in neither a commensal relationship with the fish nor a parasitic one, it is suggested that fish play a passive role in the distribution of microorganisms present in the lake. The microbial populations removed from fish by swabbing are not truly periphytic because they are not firmly attached or embedded in the tissue but are in the slime, which is easily removed by washing or rubbing. Perhaps these organisms should be classified as "Aufwuchs"—organisms attached to but not penetrating the substratum (14,

17, 2). The proper categorical designation of these organisms is outside the scope of this work, but it is obvious that the fish sampled carried a considerable population on their surfaces which could through subsequent distribution and redistribution, depending upon the feeding and ranging habits of the fish, affect the microbial population of the water and bottom mud of the lake.

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FACTORS AFFECTING THE VIABILITY OF AIR-BORNE BACTERIA

IV. THE INACTIVATION AND REACTIVATION OF AIR-BORNE SERRATIA MARCESCENS BY ULTRAVIOLET AND VISIBLE LIGHT[1]

S. J. WEBB[2]

Abstract

The effect of ultraviolet and visible light on air-borne *Serratia marcescens* has been studied. It was found that prolonged exposure to 3400–4500 Å and 5200–5800 Å radiations can be lethal to air-borne cells. Also, both wavebands were able to photoreactivate cells irradiated with U.V. light of 2800–3200 Å. The U.V. exponential death rate was not observed with visible light and a lag phase of from 15 to 20 minutes was encountered before any deaths occurred. Red dyes, of the alizarin type, were found to desensitize cells to U.V. irradiation damage, whereas blue and yellow dyes sensitized them. The effects of U.V. and visible light were additive when used simultaneously, but reactivation occurred when cells were exposed to the visible light immediately after U.V. irradiation. A mechanism, based on the long-distance transfer of excitation energy, is outlined and offered as a possible explanation for these experimental results, and for those of other workers on the inactivation and photoreactivation of microorganisms.

Introduction

Much of the work on the action of light on bacterial cells has been conducted with suspensions of cells in water or films on the surface of agar. Although there has been extensive work on the effects of environment on bacterial aerosols in the dark, little has been reported on the behavior of air-borne cells exposed to ultraviolet (U.V.) and visible light. The work reported on this topic (reviewed by Wells (17)) has mainly concerned itself with the ability of wavelengths between 2500 and 2800 Å to destroy cells. However, Wells and Wells (16) and Koller (6) in studying radiation effects were able to demonstrate that air-borne cells were more resistant to U.V. radiation damage when suspended in air of a higher relative humidity (R.H.) than in air of lower R.H. and also that air-borne cells were more sensitive to U.V. than cells in water.

To ascertain the mechanism by which U.V. destroys cells, many workers have attempted to correlate the absorption band of the cell components with the relative lethality of various wavelengths, and since U.V. of 2650 Å appeared to be the most lethal, the nucleic acids were considered as the absorbing and damaged components. More recent studies on the kinetics of radiation deaths have indicated that several processes may be involved (Zelle (18)). As to the exact cause of death, some workers believe it to be due to the production of a substance poisonous to the cell, probably hydrogen peroxide. However, others like Lea and co-workers (7) maintain that the kinetic and temperature

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coefficients characteristic of death by irradiation do not conform to those of death by disinfection, and suggest that death by irradiation is probably due to the disruption of a vital molecule. Similarly workers studying the photoreactivation of irradiated cells have assumed that death results from the production of a toxin by one wavelength and its destruction by another, or, alternatively, that reactivation does not affect the U.V. damage but avoids its consequences by initiating the synthesis of a substance which will act as an alternative metabolite. Both theories require the participation of a photochemical synthesis which either blocks or frees a particular metabolic pathway. It is the purpose of this paper to describe experiments on the inactivation and reactivation of air-borne *Serratia marcescens* by various U.V. and visible wavelengths and to offer an alternative mechanism governing these processes.

Materials and Methods

I. Preparation of Aerosol Suspensions

Suspensions of *Serratia marcescens* containing approximately 1×10^{10} cells/ml were made in a medium of 1% Bacto-protone plus 1% Difco yeast extract by adding, in a 5% weight-to-volume ratio, frozen pellets* of the organism. In order to stabilize the cells to aerosolization and air-storage, 6% inositol was added (15). Twenty-milliliter aliquots were used in a Collison spray to generate the aerosol and the aerosol carried into a 1100-liter rotating drum. The drum was similar to that described by Goldberg *et al.* (3) but had its circumference made of a plastic that would transmit light of 2800 Å and above. When dyes were used they were added to the 20-ml aliquot of suspension in a 0.5% weight-to-volume ratio and adjusted to pH 7, 10 minutes before atomization. All aerosols were held at 30% R.H. and 25° C.

II. Light Sources

(*a*) General Electric R.S. sunlamps with a spectrum of 2800–7500 Å. These lamps generate about 1.7 w in the 2800–3200 Å region and 7.0 w in the 4000–6000 Å region.

(*b*) Germicidal lamps generating 0.06 w of 2800–3200 Å. The lower wavelengths were absorbed by the drum plastic.

(*c*) Fluorescent daylight lamps having a spectrum of 3500–7500 Å with an emitted peak of 8 w in the 5500–5800 Å band.

III. Filters

Light filters were made by placing a 0.5% aqueous solution of a dye in a 9×9×1/4-in. tray made of 1/8-in. glass. The light path through the filters was therefore 0.5 in. with 0.25 in. through the filtering dye solution. The transmittance of each solution was measured by a Unicam spectrophotometer.

IV. Assessment of Viability in the Aerosols

Samples of aerosols were taken at 5-, 10-, or 15-minute intervals (depending on the time the aerosol was to be stored) in Shipe impingers containing 10 ml of 0.85% NaCl. Viable counts were then made by the drop plate method on Tryptose agar. These recoveries were compared with those obtained when aerosols were generated from 6% inositol and stored in complete darkness.

*Frozen pellets of the organism were supplied by the U.S. Army Chemical Corps.

TABLE I

The effect of various stains on the survival of *S. marcescens* exposed to germicidal and sunlamp radiations

Compound		Transmitted wavelength, Å	No. of cells/liter of air after various times of exposure				
			15 min	30 min	60 min	120 min	120 dark
Alizarin red S.	U	6500–8000	1.6×10^{6}	1.3×10^{6}	1.3×10^{6}	9.2×10^{5}	1.1×10^{6}
	S		7.1×10^{5}	2.5×10^{5}	4.0×10^{4}	8.0×10^{2}	1.0×10^{6}
Orange G.	U	6500–8000	1.4×10^{6}	1.2×10^{6}	1.0×10^{6}	8.6×10^{5}	1.2×10^{6}
	S		6.3×10^{5}	1.2×10^{5}	2.1×10^{4}	5.7×10^{2}	1.4×10^{6}
Alizarin yellow G.G.	U	5200–5800	Nil	Nil	Nil	Nil	1.2×10^{6}
	S		Nil	Nil	Nil	Nil	1.5×10^{6}
Alizarin cyanone green	U	5200–5500	5.2×10^{3}	2.5×10^{2}	Nil	Nil	1.3×10^{6}
	S		Nil	Nil	Nil	Nil	1.5×10^{6}
Alizarin blue G.S.	U	4000–6000	Nil	Nil	Nil	Nil	1.2×10^{6}
	S		Nil	Nil	Nil	Nil	1.1×10^{6}
Chlorazol black P.B.	U	3400–4500	Nil	Nil	Nil	Nil	1.6×10^{6}
	S		Nil	Nil	Nil	Nil	1.4×10^{6}
No dye	U	2800–3200	5.2×10^{4}	1.6×10^{3}	Nil	Nil	1.3×10^{6}
	S	2800–7500	1.2×10^{2}	Nil	Nil	Nil	1.5×10^{6}
Fire orange A-14	U	6500–8000	5.7×10^{4}	1.8×10^{3}	Nil	Nil	1.3×10^{6}
	S		1.8×10^{2}	Nil	Nil	Nil	1.2×10^{6}
Saturn yellow A-17	U	5000–6000	5.9×10^{4}	1.6×10^{3}	Nil	Nil	1.5×10^{6}
	S		1.1×10^{2}	Nil	Nil	Nil	1.3×10^{6}

U = Ultraviolet light.
S = G.E. R.S. sunlamp

Results

It was found that under irradiation from one germicidal lamp (0.06 w) the number of viable cells per liter of air fell from approximately 2×10^6 to 10 cells in 1 hour, whereas no viable cells remained after an exposure of 30 minutes to the R.S. sunlamp radiations. When dyes were added to the aerosol the pattern of events was changed. Dyes transmitting light above 6500 Å almost completely inhibited the action of the germicidal U.V., but those transmitting lower wavelengths appeared to enhance U.V. action with peaks in enhancement being produced by 5500–5800 Å and 3400–4000 Å light. Similar results, with respect to wavelength, were obtained when the germicidal lamp was replaced by the sunlamp, but cell death was more rapid and the protective effect of the red dyes much less pronounced (Table I).

The most significant feature of these experiments seemed to be the failure of the insoluble dyes to either sensitize or desensitize the air-borne cells to the

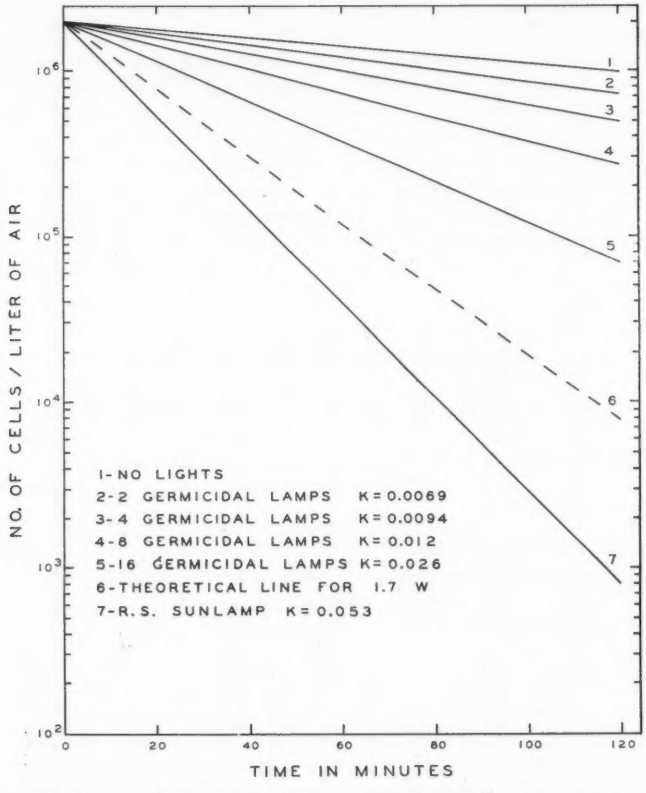

FIG. 1. The effect of increasing the intensity of 2800–3200 Å light on air-borne cells of *S. marcescens* containing alizarin red.

radiations and this was taken to indicate that the action of the dyes was from within the cell.

Since the R.S. sunlamp emitted 1.7 w of 2800–3200 Å light as opposed to the 0.06 w of the germicidal lamp, it was decided to examine the effect of increasing intensities by employing batteries of 2, 4, 8, and 16 germicidal lamps and to irradiate air-borne cells containing the dye alizarin red. From the results, shown in Fig. 1, it appeared that the death rate constants, calculated from the slope of the lines, increased by a factor of 0.0025 for an increment of 0.12 w in the intensity of the incident 2800–3200 Å light. If this trend had continued up to 1.7 w, then the death rate constant of cells produced by the sunlamp should have been about 0.041 min^{-1}. It was in fact much higher, having a value of 0.053 min^{-1}, indicating that visible wavelengths above 3200 Å were also destroying cells. Alternatively, because of the increased intensity, the red dye had reached the limit of its absorbing capacity and more damaging U.V. was reaching the vital site in the cell.

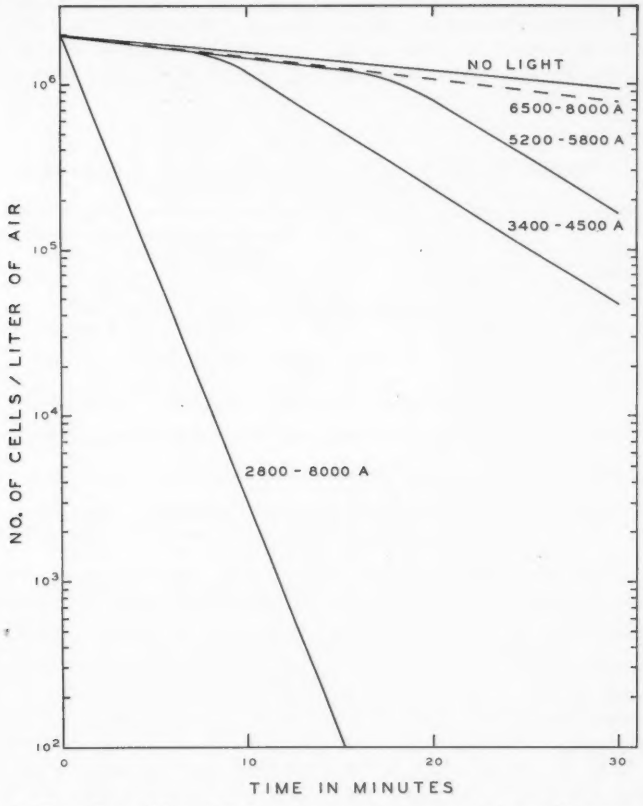

FIG. 2. The effect of filtered R.S. sunlamp radiations on air-borne *S. marcescens*.

To test these hypotheses, unprotected air-borne cells were exposed to the sunlamp radiations filtered through a solution, in water, of various dyes. The result demonstrated that visible light below 5800 Å was able to destroy cells, although the shape of the death curves changed from the exponential U.V. type. A lag phase, during which no death occurred, was evident and the duration of this phase became longer as the wavelength increased (Fig. 2). In order to ensure that these results were not due to small amounts of 2800–3200 Å light penetrating the dyes, the experiments were repeated using daylight fluorescent lamps emitting radiations of 3800–7500 Å with a peak of 8 w in the 5800 Å region and similar results were obtained. Under the conditions of these experiments there seemed to be no doubt that prolonged exposure to visible light was lethal to air-borne cells. It was again noticed that a greater number of deaths occurred, in a given time, when cells were irradiated with 3400–4500 Å or 5200–5800 Å light, than with the 4500–5200 Å wavelengths. However, the difference between the action of 4500–5200 Å and 5200–5800 Å light was small and could not reasonably be considered significant. When cells containing a dye were exposed to visible radiation it was found that both blue and yellow dyes sensitized the cells as they had for U.V. irradiation, but sensitization was mainly due to a decreased lag period. Red dyes were protective (Table II).

TABLE II

The effect of staining on the sensitivity of air-borne *S. marcescens* to 3400–4500 Å and 5200–5800 Å light

Incident wavelength (Å)	Staining dye	No. of cells/liter of air after various times of exposure to light			
		15 min	30 min	45 min	60 min
5200–5800	Nil	1.2×10^{6}	1.7×10^{5}	2.1×10^{4}	7.9×10^{3}
	Alizarin red S.	1.6×10^{6}	1.4×10^{6}	1.2×10^{6}	1.3×10^{6}
	Alizarin yellow G.G.	5.8×10^{5}	3.2×10^{4}	6.4×10^{3}	2.1×10^{3}
	Alizarin blue G.S.	5.6×10^{5}	4.1×10^{4}	5.6×10^{3}	1.4×10^{3}
3400–4500	Nil	5.1×10^{5}	4.8×10^{4}	3.6×10^{3}	3.1×10^{2}
	Alizarin red S.	1.8×10^{6}	1.5×10^{6}	1.4×10^{6}	1.1×10^{6}
	Alizarin yellow G.G.	8.2×10^{4}	5.7×10^{3}	7.1×10^{2}	—
	Alizarin blue G.S.	7.5×10^{4}	5.9×10^{3}	8.3×10^{2}	—

When the cells were exposed to the radiations from the germicidal lamp together with the filtered sunlamp radiations the effects of each were found to be additive between wavelengths of 3400–4500 and 5500–5800 Å (Fig. 3). To ascertain whether the additive effect of the wavelengths could be produced by irradiation at different time intervals, aerosols of *S. marcescens* were held at 30% R.H. for 30 minutes in the dark and then exposed to 2800–3200 Å light from the germicidal lamp for 5 minutes. Immediately afterwards, or

after intervals of 1 to 5 minutes of darkness, the air-borne cells were irradiated with visible light of 6500–8000 Å, 5200–5800 Å, or 3400–4500 Å wavelengths.

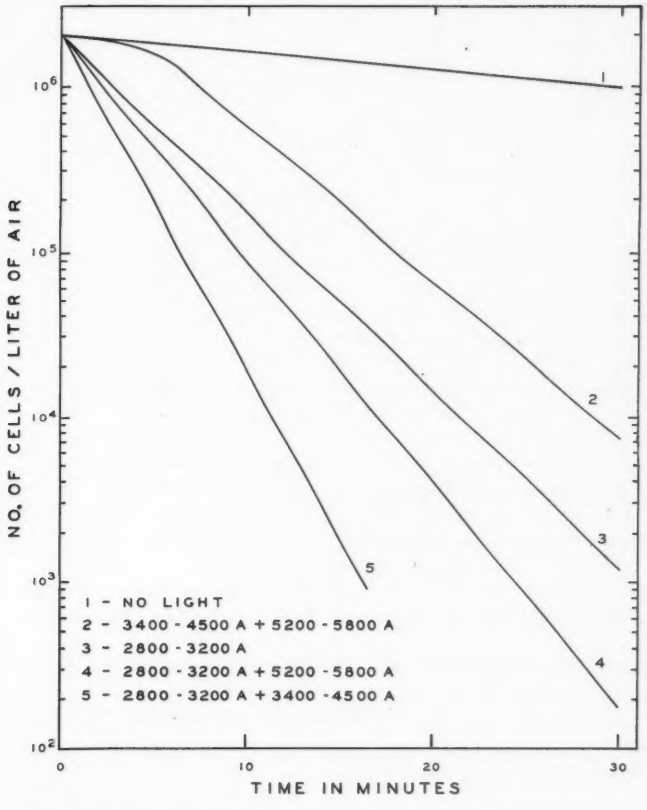

FIG. 3. The effect of exposing air-borne *S. marcescens* to 2800–3200 Å light together with 6500–8000 Å, 5200–5800 Å and 3400–4500 Å light.

It was expected that the additive lethal action of the wavelengths would be dependent on the time interval between irradiations but the results obtained were in direct contrast to these expectations. When U.V.-irradiated cells were exposed to light of 3400–4500 Å or 5200–5800 Å immediately after the U.V. light, more viable cells were recovered than with simultaneous irradiations. This increase in survival depended on the wavelength used: 3400–4500 Å light increased survival from approximately 3.0% to 30.0%, and 5200–5800 Å light from 3.0% to 10.8%. Red light was unable to produce any effect (Fig. 4). These results suggested very strongly that the phenomenon of photoreactivation was operative in aerosols and that the reactivation was extremely rapid.

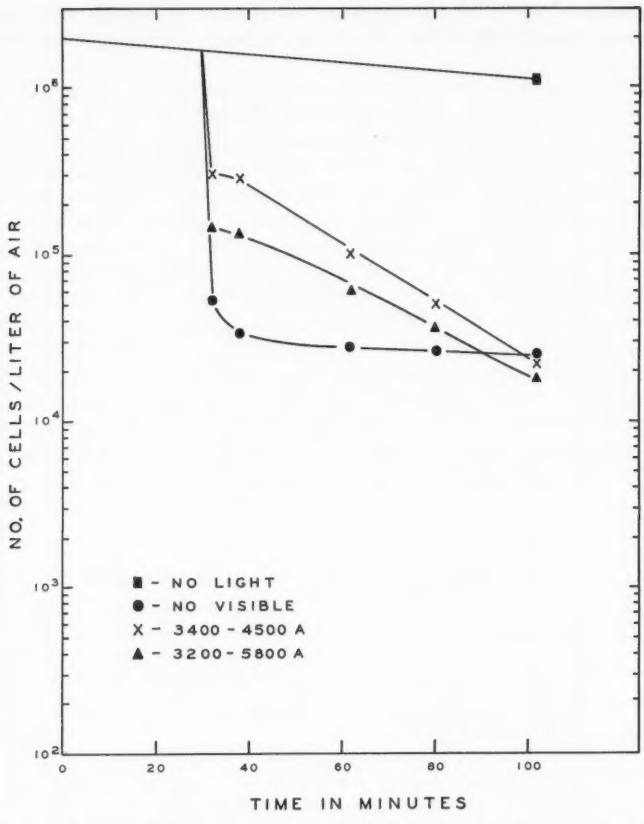

FIG. 4. The effect on air-borne *S. marcescens* of 5 minutes' irradiation with 2800–3200 Å light followed immediately by visible light.

Also, two other effects were apparent: first, that the effect of U.V. continued for a short period after U.V. irradiation had ceased, and second that subsequent deaths, after reactivation and under continuous exposure to the reactivating light, took place at a rate comparable with that produced by the reactivating light alone but without a lag phase. To ascertain the effect of dyes on this reactivation, cells stained with the blue and yellow alizarin dyes were subjected to the same treatment. It was found that staining increased the percentage of cells reactivated (Table III).

When a period of from 1 to 5 minutes of darkness elapsed before irradiating the aerosol with reactivating light, a reduced number of reactivations could be achieved with 3400–4500 Å light after a 1-minute interval of darkness. If the period of darkness was extended or the longer visible wavelengths used to reactivate, reactivation failed to take place.

TABLE III

The effect of dyes on the photoreactivation of U.V. irradiated air-borne *S. marcescens*

Reactivating wavelength (Å)	Percentage viability after a 5-min U.V. irradiation and a 5-min reactivation period		
	No dye	Blue	Yellow
None	3.2	1.2	2.5
3400–4500	29.6	44.3	41.2
5200–5800	9.7	17.4	18.3

Discussion

Visible light can be lethal to air-borne cells if the exposure time is sufficiently long, and staining the cells prior to irradiation can either sensitize or desensitize the cells to the radiations. The present work agrees in part with that of Kaplan (4), who found stained cells more sensitive to visible radiations. However, it seems that the action of the dye is in some way connected with its absorption spectrum since red dyes, of the alizarin type, acted as desensitizing agents. One might assume that the desensitizing was due to the ability of the dyes to absorb all the lethal wavelengths, including the visible. Two factors argue against this: first, the inability of insoluble red dyes to protect cells and second, that although yellow dyes would absorb U.V. and reduce its intensity, cells stained with yellow dyes were killed more rapidly than unstained ones. It is considered more probable that the dyes' action is the result of both the absorption of radiant energy and their combination with some component of the cell.

Several interesting points have arisen from the present study and it would seem pertinent to enumerate them in order that a possible mechanism may be explained:

1. The exposure time required to kill cells by irradiation with light increases as the wavelength increases. Also, with visible light there is a lag phase before radiation damage appears in the form of cell death and this phase increases as the radiant energy decreases.

2. Reactivation after U.V. irradiation is only brought about by wavelengths which are themselves lethal.

3. By decreasing the wavelength of the reactivating light, the extent of reactivation is made larger, and the time during which reactivation will occur is extended.

4. The death rate of cells after reactivation, on continuous exposure to the reactivating light, is the same as when exposed to the reactivating light alone.

It would appear that conditions which favor death by U.V. irradiation also favor reactivation. At cold temperatures and low R.H., cells die more rapidly than under the opposite conditions (1); yet their ability to be reactivated can be maintained by chilling and it is evident from comparison of the present work with published data (1, 18) that reactivation is far more rapid with air-borne cells than with suspensions in water. The necessity for dyes to enter the cell before being effective, the reactivation by light which is itself lethal, the greater reactivation when conditions favor a faster death rate under

U.V. irradiation, and the fact that reactivation has not yet been achieved chemically seem to point to a physical phenomenon, rather than chemical, with more than one stage.

It is certain that some part of the cell is destroyed by the absorption of radiant energy. However, the absorbing site need not be the one destroyed, nor does the destruction of biological activity necessarily require the rupture of a molecule into fragments or the formation of ions. Stereochemical changes, similar to those reported by Lewis *et al.* (8) for stilbine, might well serve to destroy or change biological activity. The rearrangement of protein molecules by radiant energy has been studied by McLaren (11), who found that the quantum yield increased with decreasing frequency, decreased with increasing size of molecule, and was larger for absorbed films than water solutions. If one argues that death of the cell is produced by such rearrangements, then these observations will support those on the frequency effects on bacterial cells.

When a site absorbs radiant energy, it is raised from a ground state to a higher electronic level and the absorbed energy may be dissipated by direct dissociation of the molecule, by fluorescence, or by the migration of the excitation energy to another group or molecule. That both fluorescence and migration occur in large molecules has been demonstrated by Shore and Pardee (13, 14), who showed that 2800 Å light absorbed by the aromatic acids of a protein caused the dye, in a protein dye complex, to fluoresce. Also light absorbed in the tryptophane and tyrosine residues of ovalbumin causes the protein to fluoresce (12). That a single bond may be broken by absorption of different wavelengths at different sites and that energy transfers over distances of up to 50 or 100 Å can occur has been shown by Franck and Livingstone (2) and Livingstone (9). From these latter investigations, it appears that light of 2800 Å or 5400 Å will cause CO to be released from a myoglobin–CO complex, although 2800 Å light is absorbed by the tyrosine residues and the 5400 Å light by the heme moiety.

The mechanism by which excitation energy migrates in large molecules is a matter of speculation. Such molecules usually have high electrical polarizabilities and loosely held electrons which can be conducted throughout the system in a metal-like fashion. Generally they have low-lying, excited states and exhibit poor crossing to the ground state. However, crossing may be good among upper electronic levels. It is possible that in these large molecules transfers of energy may be by both the short-range mechanism and long-range mechanism. Long-range crossings rely on dipole–dipole interaction, corresponding to excitation by electromagnetic fields, and therefore only in systems where the fluorescent band of the primary excited state overlaps the absorption band of another will transfers take place (10). Based on the experimental results of the work reported in this paper and the fact that both absorption at different sites and energy migration can occur, the scheme shown diagrammatically in Fig. 5 is offered as a possible mechanism of both inactivation and reactivation.

Sites 1, 2, and 3 are intended to represent groups or residues of a long-chain protein or nucleoprotein. When the molecule is irradiated with 2800–3200 Å light, site 1 becomes excited and transfers the excitation energy via site 2 to

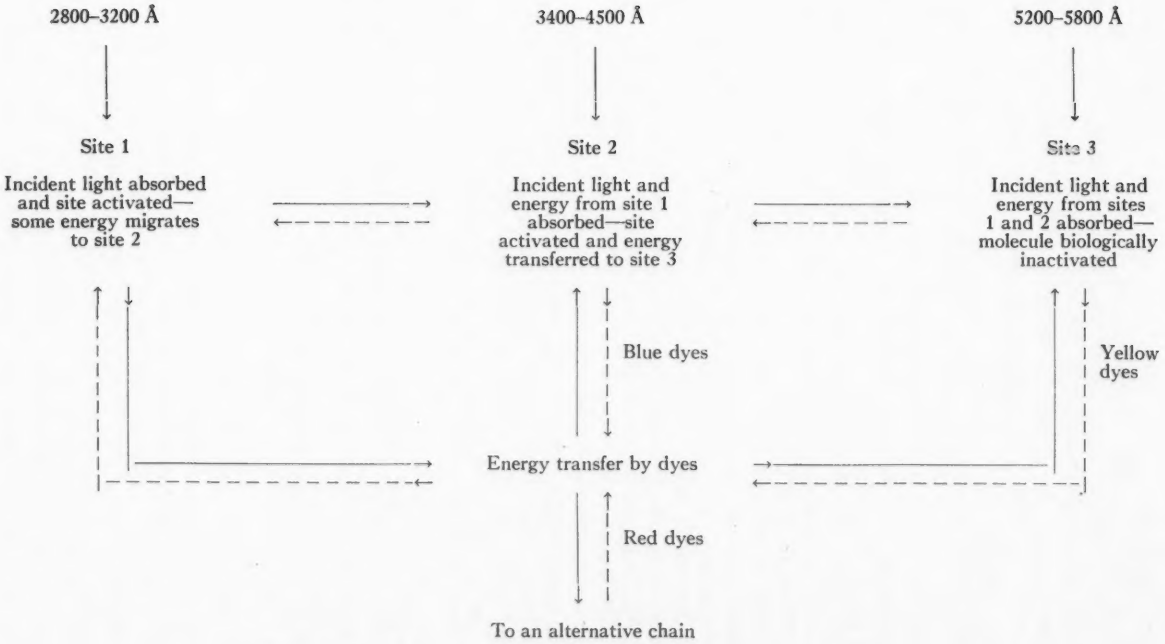

FIG. 5. A possible scheme for the transfer of absorbed radiant energy.

site 3 and, in the process, sites 2 and 3 become excited. However, visible light of 3400–4500 or 5200–5800 Å will only excite sites 2 and 3, or site 3 respectively. The energy levels of the three wavebands are such that if it was necessary for the energy to build up before becoming localized in vibrations capable of destroying site 3, or accumulate faster than it can be dissipated, the higher energy shorter wavelengths would kill cells more rapidly than the longer wavelengths. The necessity for energy to build up before becoming lethal could account for the observed lag phase before radiation damage, due to visible light, became apparent.

It is assumed that the energy transfers take place only when neighboring sites reach a certain level of excitation, and that transfers are by short-distance mechanisms together with the long-distance mechanisms made available by the overlapping of the fluorescent band of site 1 and the absorption band of site 2, and so on. With this system in mind, it becomes possible that reverse energy migrations may occur, but only when the sites are activated. That is to say, if an U.V.-irradiated molecule is exposed to visible light immediately after the removal of the U.V. light, site 1 is still activated and will receive energy from either site 2 or 3 and thus relieve site 3 of excess energy. If, however, sufficient time has elapsed to allow site 1 to revert to another electronic state, these reverse transfers will become impossible and reactivation will not occur. One would expect then that the higher energy reactivating light would produce a greater number of reactivations since it would supply more energy to site 1 and allow it to remain longer in a particular excited state in which it would accept energy from sites 2 or 3. Once the sites had reached an electronic state disallowing the reverse transfer of energy, the death rate of cells would automatically revert to that associated with the reactivating light itself but without a lag phase. In any given, large, conjugate molecule, such as a nucleoprotein, there must be many sites capable of various excited electronic states that will allow such energy transfers. For simplicity in the present scheme, only three are postulated, but in all probability there are many more.

The role of the dyes can be explained on the same basis. Red dyes would be expected to have fluorescent bands above 6500 Å and thus energy transferred to them from any site or obtained by direct absorption could not be passed to site 3. The energy must either be transported along an alternative chain or be dissipated by harmless fluorescence; in either case the excitation energy from site 1 is split into two portions and cell protection will result. The degree of protection, however, will still depend on the intensity of the incident radiations. Blue and yellow dyes should fluoresce within a band of 3500–6000 Å; thus these dyes would not only act as transfer agents for the excitation energy from site 1 or 2, but also transfer energy they themselves gain by the absorption of U.V. or visible light, hence cell deaths will occur more rapidly. The system outlined, assuming energy transfer in an excited conjugate system to be reversible, can explain many of the phenomena found to be associated with photoinactivation and photoreactivations. It follows that the various conditions (such as temperature, water content, pH, wavelength of light, or staining) which favor inactivation of a cell by U.V. or visible light must produce conditions in the system favoring energy migrations and, in doing so, must also

favor reverse migration and the consequent reactivation. The fact that oxygen appears necessary for inactivation and reactivation might well be due to its ease of activation by radiation and that in its excited form as molecules, atoms, or hydrogen peroxide it is part of the energy transfer mechanism. Therefore it becomes unnecessary to think of hydrogen peroxide acting as a disinfectant or to postulate the photoformation and destruction of toxins or the photosynthesis of some alternative metabolite.

Acknowledgment

I express my thanks to Mr. C. Watson for his valuable technical assistance during this work.

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FACTORS AFFECTING THE VIABILITY OF AIR-BORNE BACTERIA

V. THE EFFECT OF DESICCATION ON SOME METABOLIC SYSTEMS OF ESCHERICHIA COLI[1]

S. J. WEBB[2]

Abstract

The effect of desiccation on the ability of *Escherichia coli* to carry out oxidations, decarboxylations, and adaptive enzyme synthesis has been studied. Changes in cell metabolism due to drying were studied; and also the influence on such changes of protecting the cells with inositol. Dehydration brought about the release of 260-mμ absorbing material from the cell, the amount released being increased in the presence of inositol. Dehydration reduced amino acid oxidation and increased decarboxylation and glucose oxidation. Protecting the cells against death with inositol not only failed to prevent these changes but, with the exception of glucose oxidation, made them more pronounced. The synthesis of adaptive β-galactosidase was found to be greatly inhibited by desiccation and this inhibition could be prevented by inositol. It is suggested that a structural change in the nucleoproteins concerned in protein synthesis is responsible for the death of dried cells rather than damage to their membrane.

Introduction

In earlier work on the effects of relative humidity (R.H.), temperature, and chemical additives on the death of air-borne bacteria, it was found that not only did each factor affect the behavior of the air-borne cells, but the effect of each factor could be modified by the simultaneous presence of another (1, 2, 3). Chemical additives were found able to control and alter the reaction of the cells to their environmental conditions of R.H. and temperature. The action of these chemicals appeared to be associated with their structure: those possessing a six-membered ring nucleus with >CHOH and >C—NH_2 group were able to protect cells against damage in aerosols. Inositol proved to be the most efficient stabilizer. As a result of this work, it was considered that the death of air-borne cells resulted from a structural change in one of their vital components when "bound" water was removed. The protective compounds, such as inositol, were considered to be able to take the place of water molecules during periods of desiccation and maintain the natural structure of the vital component. Because of the aerosol stability of some types of bacteria which contain a high proportion of inositol in their natural structure, and the instability of lipoprotein to desiccation, the cell membrane was suggested as the vital component undergoing damage in aerosols. It seemed reasonable, therefore, to follow this work by examining the physiological changes produced in cells by aerosolization. However, as it proved impossible to collect a sufficient number of cells from an aerosol for physiological study, it was necessary to simulate aerosol damage by desiccation at

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various R.H. levels. It is the purpose of this paper to describe experiments which have led to the conclusion that the destruction of the biological activity of a component concerned in the synthesis of protein is the cause of cell death in aerosols and not membrane damage.

Materials and Methods

I. Organism

Escherichia coli Type 1, obtained from the University of Alberta. In all experiments 24-hour cultures, grown at 37° C on a rotary shaker, were used. The growth medium was determined by the enzyme system studied.

II. Desiccation and Preparation of Cell Suspensions

After 24 hours' growth, 20-ml aliquots of the culture were removed. The cells were sedimented and washed twice in distilled water by centrifugation at 8000 r.p.m., and then resuspended in 20 ml of water, or a 6% solution of inositol in water. After 10 minutes' incubation at 37° C, the cells were sedimented into a pellet or filtered onto a millipore filter. The cells were then transferred to a drying cabinet maintained at 30% R.H. and 25° C by circulating air. Drying was carried out for periods of up to 5 hours, after which the cells were resuspended in 20 ml of buffer for viable counting and enzymic tests.

III. Analysis of Supernatant Fluids of Dried and Nondried Cells by Ultraviolet Spectrophotometry

At the end of a 5-hour period of drying at 30% R.H., the cells were washed from the millipore filters with 20 ml of sterile distilled water. The cells were then removed by centrifugation and 2-ml aliquots of the supernatant fluid were placed in quartz cuvettes and their ultraviolet (U.V.) absorption spectrum determined with a Unicam Spectrophotometer. This spectrum was then compared with that of the supernatant from the third washing of fresh cells.

IV. The Oxidation of Sugars and Amino Acids

The organism was grown for 24 hours in a medium of 1% Bacto-tryptose and 1% Casein hydrolyzate at pH 7.2. The cells were then harvested and dried as in II above and finally resuspended in 20 ml of 0.2 *M* phosphate buffer, pH 7.2. Two-milliliter aliquots of these suspensions together with 0.2 ml of a 1% solution in buffer of a sugar or amino acid were used in a Warburg vessel. Oxygen uptake at 30° C was measured by the standard Warburg technique.

V. The Decarboxylation of Amino Acids

The organism was grown for 24 hours in 1% Bacto-tryptose, 1% Casein hydrolyzate, 1% glucose, and 0.005% niacin, pH 6.5. Cells were harvested, dried as in II above, and resuspended in 20 ml of phosphate buffer, pH 4.8. Carbon dioxide evolution from 0.2 ml of a solution of the amino acid in buffer was measured by the standard Warburg technique.

VI. The Synthesis and Activity of Adaptive β-Galactosidase

Two sets of experiments were carried out: (*a*) with preadapted cells and (*b*) with nonadapted cells.

(*a*) *Preadapted Cells*

The cells were adapted by growth for 24 hours at 37° C in a medium of 1% Bacto-tryptose + 1% Casein hydrolyzate + 1% lactose, pH 7.0. After growth the cells were harvested and dried as in II above and resuspended in 16.0 ml of 0.2 *M* phosphate buffer, pH 7.2. To this suspension, or to the supernatant obtained by removing the cells by centrifugation and filtration, 4.0 ml of a 0.01 *M* solution, in buffer, of orthonitrophenol-β-D-galactopyranoside was added, and the tubes incubated in a water bath held at 37° C. Immediately, and at subsequent intervals of 5 minutes, 1.0-ml aliquots were removed and placed into 9.0 ml of 0.1 *M* sodium carbonate and the cells removed by filtration through a millipore filter. The amount of orthonitrophenol released was calculated from the optical density of the filtrates at 420 mμ using a Unicam Spectrophotometer and a standard graph of the optical density of various concentrations of orthonitrophenol in sodium carbonate. The enzyme activity was then compared with that of nondried cells.

(*b*) *Nonadapted Cells*

The organism was grown at 37° C in a medium of 1% Bacto-tryptose + 1% Casein hydrolyzate + 1% glucose, pH 7.0, and after 24 hours' growth the cells were harvested and dried as in II above. The dried cells and washed, nondried cells were resuspended in 20 ml of fresh medium containing 1% lactose instead of glucose, and transferred to a water bath at 37° C. Immediately, and at subsequent hourly intervals, the cells were sedimented and washed once in distilled water by centrifugation and then resuspended in 16 ml of phosphate buffer and 4 ml orthonitrophenol-β-D-galactopyranoside solution and the enzyme activity assayed as in VI(*a*).

Results

A large difference was found in the behavior of cells desiccated by the two methods. When the cells were dried in the pellet form in 50-ml centrifuge tubes, deaths were fewer in those sedimented from distilled water than in those from 6% inositol. Under these conditions, inositol was toxic to the cells, which was a complete reversal of its action in aerosols. However, if the cells were desiccated in films on millipore filters, their behavior simulated that in aerosols so this technique was used for the subsequent studies (Table I).

TABLE I

The effects of different methods of drying on *Escherichia coli*

Method of drying	% viable recovery after periods of drying of:					
	5 min	1 hr	2 hr	3 hr	4 hr	5 hr
Aerosol (H_2O)	20.1	3.6	1.2	0.7	0.5	0.4
Pellet (H_2O)	73.6	52.1	53.6	51.1	50.7	53.7
Film (H_2O)	24.2	3.1	0.8	0.8	0.7	0.6
Aerosol (inositol)	84.2	83.1	75.3	72.4	68.7	65.7
Pellet (inositol)	70.6	10.2	0.9	0.8	0.8	0.7
Film (inositol)	78.3	76.2	73.3	69.1	63.3	57.8

It was considered that in the pellets, due to the formation of a skin of dead cells, drying was incomplete or extremely slow and that this resulted in the production of damaging, high osmotic pressures when inositol was present. This would not occur in aerosols or quickly dried films.

It was expected that by protecting the cells with inositol any leakage of cell material from the dried cells would be prevented, but the results obtained were in direct contrast to these expectations. In Fig. 1, the ultraviolet absorption spectra of the supernatant fluids of dried and nondried cells are shown, and it can be seen that, although the over-all spectra of fluids are the same, considerably more 260-mμ absorbing material was released from the inositol-protected cells than from those dried from water. The reason for this was difficult to visualize and, as many more of the protected cells remained viable, it was considered that the leakage of this material was not related to the death of the dried cell. Studies were made, therefore, on the effect of desiccation on some of the metabolic functions of the cell.

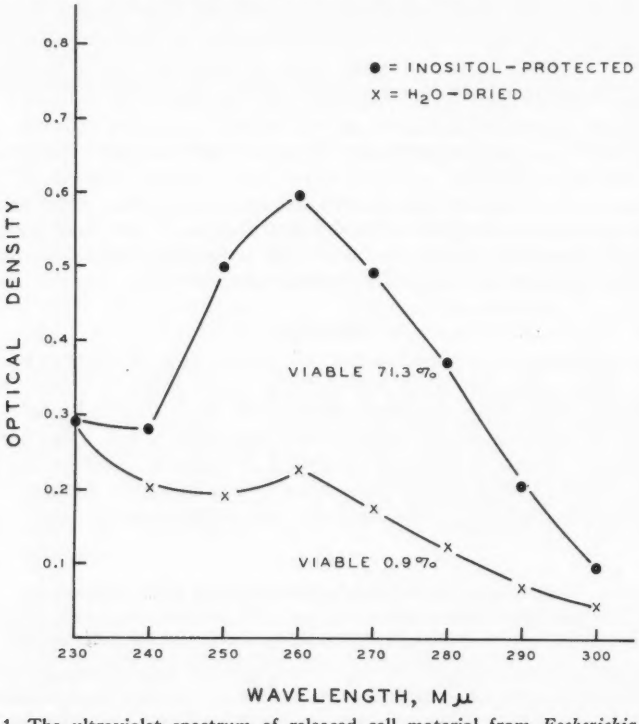


FIG. 1. The ultraviolet spectrum of released cell material from *Escherichia coli* on desiccation.

The Effect of Drying on the Ability of Cells to Oxidize Various Compounds

In Table II are tabulated the O_2 uptakes (μl/hour) of nondried cells and cells dried for 5 hours at 30% and 70% R.H. If the results are viewed in terms

TABLE II

The oxidation of various compounds by dried and nondried *Escherichia coli*

Compound	Nondried		H_2O-dried		Inositol-dried	
	μl O_2/hr	% viable	μl O_2/hr	% viable	μl H_2O/hr	% viable
			30% R.H.			
L-Alanine	9.1	100	2.9	0.8	1.0	63.2
L-Serine	20.1	100	3.1	1.0	1.6	53.1
L-Threonine	8.1	100	1.6	1.2	1.0	58.3
Glutamic acid	3.7	100	1.1	1.6	0.8	59.2
Catechol	3.4	100	2.3	0.9	1.7	52.7
Glucose	36.9	100	16.3	0.7	19.4	54.6
			70% R.H.			
L-Alanine	9.9	100	5.3	50.4	1.2	76.3
L-Serine	20.8	100	14.6	53.2	2.4	80.3
L-Threonine	8.5	100	4.8	58.2	1.6	77.6
Glutamic acid	4.1	100	3.1	56.5	1.1	78.5
Glucose	38.3	100	40.5	59.3	28.1	73.2

of the wet weight of cells, then it appears that in all cases the rate of oxidation was greatly reduced by desiccation and still further reduced by protection with inositol. If, however, the results are analyzed on the basis of the number of viable cells remaining after the 5-hour drying period, the situation becomes more complicated. In the case of the cells dried from water, there appeared to be no correlation between the O_2 uptake of cells held at 30% R.H. and those held at 70% R.H. To obtain the O_2 uptake by the same number of cells, it is necessary to multiply the O_2 uptake of the 30% R.H., dried cells by approximately 100, and the 70% R.H. figures by 2. If this is done, then it would appear that, in the case of serine for instance, O_2 uptake had increased from 20.1 μl/hour to 310 μl/hour at 30% R.H. and from 20.8 μl/hour to 29.2 at 70% R.H. With the protected cells there was good correlation, on the viable number basis, between the O_2 uptake rates of the cells held at the two R.H. levels. However, there was a definite inhibition of amino acid oxidation, whereas glucose oxidation was slightly higher. When the protected versus the nonprotected cells dried at the two R.H. levels were compared, it was evident that a difference existed between their ability to oxidize the amino acids. Protected cells always displayed a reduced oxidation ability over nondried cells. It seemed possible that these results were due to the relative ability of the substrates to reach the enzyme systems of the cell. Thus, in inositol-dried cells their permeability to amino acids had been reduced by some combination of inositol with the membrane, and in cells dried from water, the membrane had been altered allowing the amino acids to enter more freely. It was decided, therefore, to examine the decarboxylation of amino acids to ascertain whether or not the same phenomenon occurred.

The Decarboxylation of L-*Arginine by Dried and Nondried E. coli*

Unfortunately, *E. coli* would not decarboxylate the oxidizable amino acids and it was necessary to use L-arginine for this series of experiments. In the initial studies it was found that there was a tremendous increase in arginine decarboxylation by *E. coli* on drying, with the enzyme activity increasing

from 5 μl CO_2/hour for nondried cells to 86 μl CO_2/hour with cells dried from inositol. In direct contrast to amino acid oxidation, the cells dried from water, although having a much higher activity than nondried cells, displayed a lower CO_2 evolution rate (48 μl/hour) than the protected cells. It appeared, therefore, that in this case the protected, dried cells were more permeable to arginine than the nonprotected ones. It seemed clear that the increase in decarboxylation was not correlated with viable cell numbers and that the enzyme activity could go on after the death of the cell. This assumption was tested in two ways: first, by assaying decarboxylase activity after different times of drying, and second by suspending the cells for 1 hour in different concentrations of acetone, ethanol, and phenol. In the latter case, the cells were washed twice in water to remove the chemicals and then resuspended in buffer for enzyme activity assay.

After 1 hour of drying, the arginine decarboxylation activity of cells dried

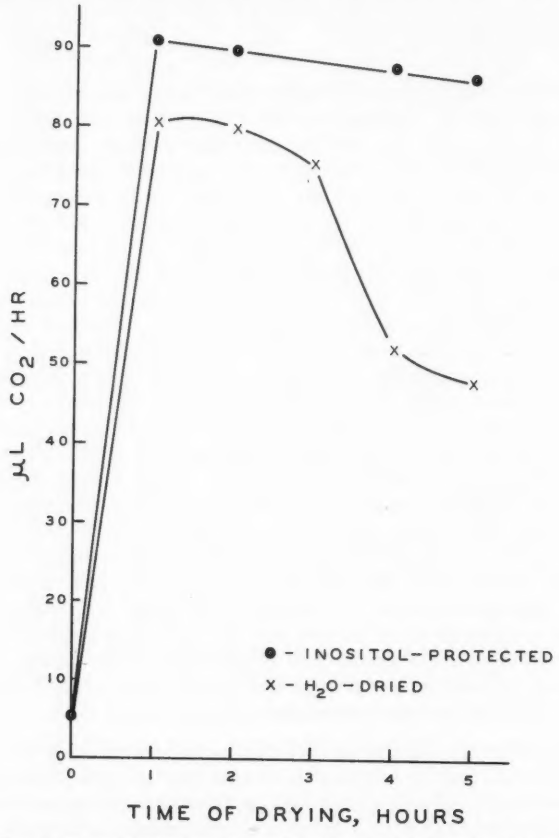


FIG. 2. Decarboxylation of L-arginine by air-dried *Escherichia coli*.

from water increased to 82 μl CO_2/hour and then gradually fell to 48 μl CO_2/hour as the drying time increased. The activity of the inositol-protected cells, however, increased to 90 μl CO_2/hour during the first hour and fell only slightly to 86 μl CO_2/hour after a 5-hour drying period (Fig. 2). The viability of the cells after the first hour of drying was considerably different: of those dried from water 3.1% were viable whereas 76.2% of the protected cells remained alive (Table I). This indicated that decarboxylation was not dependent on the viability of the cell and that contact between the enzyme and its substrate was more easily achieved in dried cells. It was possible also that the lower activity of cells dried from water was due to a release of enzyme into the discarded supernatant fluids, but tests for the enzyme in these fluids failed to demonstrate its presence. It was not clear why the lowered activity of the unprotected, dried cells should exist, for it did not seem reasonable that the permeability change, producing the high activity in 1-hour-dried cells, should revert, on continued drying, to one producing a lower activity. A more plausible answer was found when the enzyme activity of

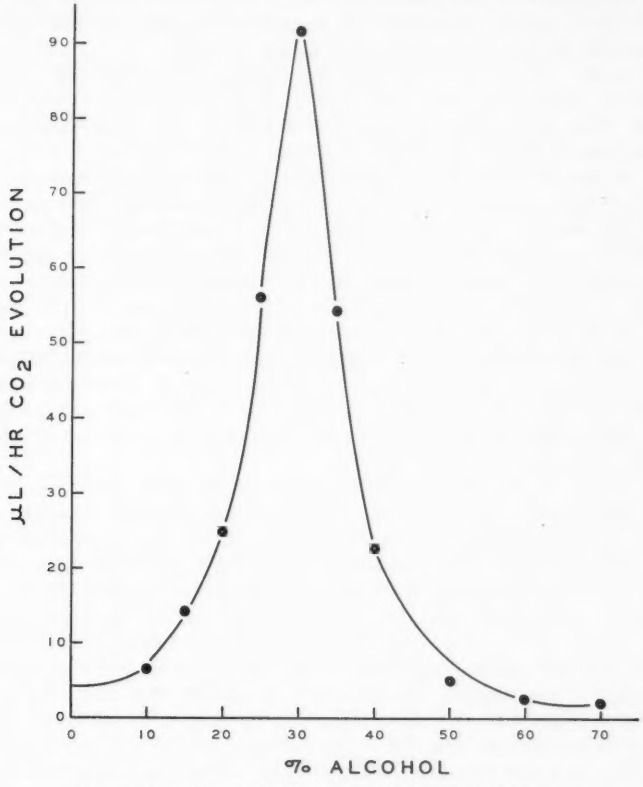


FIG. 3. Decarboxylation by ethanol-dried *Escherichia coli.*

cells physiologically dried by chemicals was studied. When the cells were allowed to incubate for 1 hour in solutions of 1.0% phenol, 25% acetone, and ethanol, only the latter two compounds were able to produce an increase in decarboxylase activity. The enzyme reaction rates obtained were 36 and 57 μl CO_2/hour respectively, and since ethanol produced the greatest change it was decided to examine the effects of alcohol concentration. In Fig. 3, the arginine decarboxylase activity of cells suspended for 1 hour in ethanol is plotted against alcohol concentration and it can be seen that concentration had a profound effect. Up to a concentration of 30%, the enzyme activity increased from 4.0 to 94 μl CO_2/hour and then rapidly decreased as the alcohol concentration increased above that level. The same phenomenon was encountered with acetone although the peak activity of 62 μl CO_2/hour was found at a concentration level of 20%. Tests for the enzyme in the alcohol supernatant by direct assay or by evaporation of the alcohol failed and it was assumed that a destruction of the enzyme took place on prolonged drying in air or enforced drying by the alcohol. It was evident that inositol could, in some way, prevent the destruction of the enzyme during air drying, but the addition of inositol to the alcohol suspensions of the cell failed to prevent their death or the inhibition of decarboxylase activity.

These results made it clear that the increase in decarboxylase activity produced by desiccation was not associated with the mechanism by which cells died. Also the results of the experiments on the effects of desiccation on amino acid oxidation and decarboxylation were in direct contrast to one another. In the former with protected cells, the enzyme activity was reduced, and in the latter, it was greatly increased; therefore unless one postulated a different mechanism governing permeability for individual amino acids in *E. coli*, the hypothesis of membrane damage being responsible for death in this case would not seem to hold. It was possible that the change in sugar metabolism reflected by the increased oxidation of glucose by cells surviving desiccation could be connected with death. Therefore, it was decided to examine this phenomenon more closely, and to utilize the enzyme β-galactosidase since this enzyme has been extensively studied. The selection of this enzyme was made also because it is adaptive in the organism under study and as such might reveal any change in the ability of the dried cell to synthesize proteins.

In the initial series of experiments preadapted cells, grown in a lactose medium, were used. The cells were dried with and without inositol and both the cells and the washing supernatants assayed for β-galactosidase activity. These activities were then compared with those obtained using nondried cells. The results resembled those on decarboxylation. Desiccation on non-protected or protected cells produced an increase in the rate of enzyme activity from 7.8 to 56.2 and 60.2 mg orthonitrophenol (O.N.P.)/hour. With this enzyme system, however, 11.6% of the total activity was present in the cell-free supernatant of nonprotected, dried cells and 22.4% in that of protected cells. The drying of nonadapted cells also increased the basal rate of β-galactosidase activity from 0.8 to 1.0 and 1.2 mg O.N.P./hour (Table III). Again desiccation had produced a change in permeability which allowed a more rapid contact between the enzyme and its substrate. The

same change, however, was evident in both protected and nonprotected cells. Since only 1.0% of unprotected cells survived drying as opposed to the 60% survival of protected cells, to follow the synthesis of this enzyme required a slight change in technique. To obtain the same number of viable cells after drying, it was necessary to dry for 5 minutes only and to use 80 ml of the original culture for unprotected drying and 24 ml for protected drying. The dried cells were resuspended in 20 ml of lactose medium, incubated at 37° C for various periods of time, and then assayed for the presence of β-galactosidase.

TABLE III

The activity of the β-galactosidase of dried and nondried cells

	Nondried	H_2O-dried	Inositol-dried
Preadapted			
Cells (2×10^9)	7.8*	56.2	60.2
Supernatant	Nil	6.5	13.6
Nonadapted			
Cells	0.8	1.0	1.2
Supernatant	Nil	Nil	Nil

*Activity expressed in mg/hour release of orthonitrophenol.

These experiments demonstrated a marked difference between protected and nonprotected cells, and a similarity between protected and nondried cells. With the nondried cells there was a lag of 80 minutes before any increase in β-galactosidase activity became apparent. During the next 60 minutes the enzyme activity increased from 0.8 mg O.N.P./hour to 6.5 mg O.N.P./hour and reached a maximum rate of 8.5 mg O.N.P./hour after 180 minutes. Protected, dried cells displayed a longer initial lag of 100 minutes, but the same rate of enzyme synthesis in the subsequent 60-minute period. With these protected cells β-galactosidase activity reached a value of 6.4 mg O.N.P./hour in 160 minutes, and a maximum rate of 8.5 mg O.N.P./hour

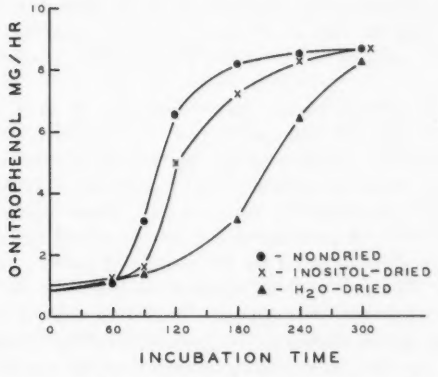


FIG. 4. The synthesis of β-galactosidase by dried and nondried *Escherichia coli*.

after 200 minutes. The sequence of events with the nonprotected cells was very different. The lag period lasted for 140 minutes and during the next hour the enzyme activity only increased from 1.0 to 3.2 mg O.N.P./hour. Enzyme synthesis, however, continued and activities of 6.4 and 8.1 mg O.N.P./hour were reached after 260 and 300 minutes respectively (Fig. 4).

Discussion

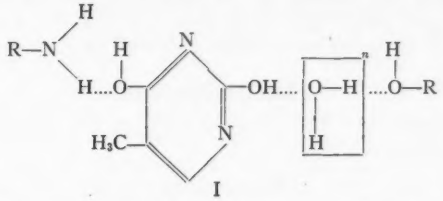
From the work described, it was evident that the desiccation of the cell results in a change of its permeability to both sugars and amino acids. However, since this change occurred in inositol-protected cells as well as nonprotected cells, the increase in permeability would not seem to be the direct cause of death.

The U.V. spectra of the supernatant fluids of dried cells demonstrated that more 260-mμ absorbing material was released from protected cells (Fig. 1). Since this release of cell material could not be produced by suspending cells in solutions of inositol, it is clear that the presence of inositol on drying causes the loss of this material and also the greater loss of β-galactosidase. The loss of a preformed enzyme or some nucleic acid substance, therefore, cannot be the cause of death, since many more of the protected cells remained viable after drying. Death could result, however, from some changes in physiological function of the cell associated with the release or retention of a cell constituent. It could be argued that the increase in oxidation of glucose as demonstrated by the few remaining viable cells following unprotected drying is indicative of a rapid and uncontrolled expenditure of energy leading to the death of some cells. Also that the large increase in decarboxylation of amino acids depletes the growth medium of essential amino acids. Several factors would seem to argue against these hypotheses. First, it would be necessary to postulate a different permeability change in the cell governing the entry of oxidizable amino acids and those undergoing decarboxylation, or a greater destruction of amino acid oxidases in protected cells. Second, the depletion of amino acids in the medium by decarboxylation would be greater with protected than unprotected cells. Third, the increase in decarboxylase or β-galactosidase activity was not related to the number of remaining viable cells since there was as big an increase in decarboxylation rate with protected 80% viable cells as with cells 100% killed by chemical dehydration in ethanol.

It is evident, on a mass basis, that a decrease in total oxidation activity takes place on drying both the protected and unprotected cells. This must mean that the enzyme system controlling the process is partially destroyed, or inhibited in some way, in both kinds of dried cells. The inhibition of amino acid oxidation could be taken to indicate that there was a metabolic change from an energy-producing process to one of increased synthesis and that the extra energy required is supplied by virtue of the greater ability of dried cells to oxidize glucose. The new system must of necessity be an organized one and it would seem reasonable to suppose that it is this secondary system of synthesis which is disorganized in nonprotected cells. Death of a cell could take place either by the mass disorganization of its constituent parts or by

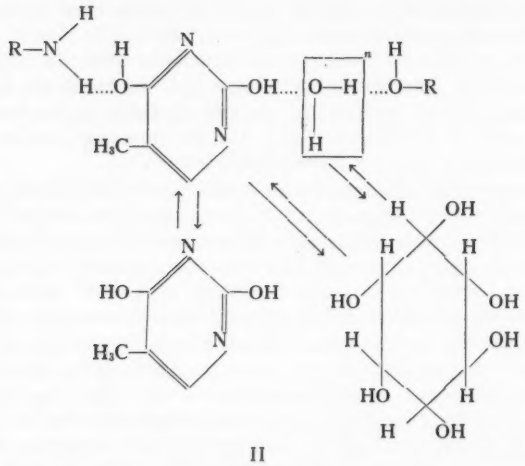
the destruction of the integrity of a single component concerned in the synthesis. The evidence that such a system is affected by desiccation is shown by the inability, or reduced ability, of dried cells to form the adaptive enzyme β-galactosidase. The fact that inositol was able to maintain this system of synthesis during periods of desiccation and also retain the viability of the cell seems to point to the destruction of the mechanisms concerned in protein synthesis as being the cause of microbial death.

In previous work (1, 2, 3) it was suggested that inositol preserved the viability of air-borne cells by replacing the "bound" water of the cell. These water molecules in the wet cell, by forming hydrogen bonds, are part of the structural framework of the cell components and it was considered that the –CHOH groups of inositol were able to form the same type of H-bonds as water and so maintain the structural integrity of some vital component. The particular component preserved was thought to be the cell membrane, but the present work indicates that this is not the case, as cell material is equally able to leach from the protected as the nonprotected cell. The loss of considerably more 260-mμ absorbing material from inositol-protected cells than from the nonprotected ones makes it appear that inositol can displace some purines or pyrimidines and, in doing so, preserves the cell's ability to carry out enzyme synthesis. It is postulated, therefore, that some of the purine and pyrimidine bases of the nucleoproteins are held in their natural position by H-bond bridges and that some of these bridges are through water molecules, as shown in simple form in scheme I below, using thiamine as an example.



I

The removal of water molecules would free one >C—OH group of thiamine allowing the molecules to rotate and, in so doing, enable the group to H-bond to another group. This new bond may be irreversible with water so that the over-all structure of the large conjugate molecule is irreversibly changed. If the H-bond of the second >C—OH group, on which rotation takes place, is weaker than those formed by inositol, then in the absence of protective water molecules, inositol may displace thiamine. This would produce the increase in 260-mμ absorbing material from protected cells. Such a system would give inositol two roles, one of replacing structural water molecules and the other of replacing structural pyrimidines. It could also explain why some pyrimidines protect air-borne cells and why the protective abilities of these compounds and inositol are found to be additive (1, 2, 3). All that is required is the movement of the pyrimidine to be dependent on the relative concentrations, within the organism of both compounds as shown in scheme II.



II

Thymine is used to illustrate the system because this compound is thought to be a cofactor in decarboxylation. Thus it is possible that its release is responsible for the observed increase in amino acid decarboxylations by dried cells, instead of the supposed change in the permeability of the cell to amino acids. To ascertain the nature of the released nuclear material requires chromatographic and electrophoric analysis of the cell supernatant fluids. This work is now in progress and will be reported in a subsequent paper.

Acknowledgment

I express my thanks to Mr. Ronald Hodges for his valuable technical assistance during this work.

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SOME OBSERVATIONS OF THE EFFECT OF FILTRATES OF SEVERAL REPRESENTATIVE CONCOMITANT BACTERIA ON CLOSTRIDIUM BOTULINUM TYPE A[1]

F. D. CRISLEY[2] AND G. E. HELZ

Abstract

Filtrates of growing cultures of *Bacillus sphaericus*, *Clostridium sporogenes*, *Escherichia coli*, and *Streptococcus faecalis* inhibited germination of spores of *Clostridium botulinum* type A. Of the four filtrates only that of *E. coli* was inactive at low (1:8) concentrations, and all were inhibitory at high (1:2) levels. Only filtrates of *B. sphaericus* and *C. sporogenes* affected lysis (increased) of washed cells of *C. botulinum*, and only *S. faecalis* filtrate altered botulinal toxigenicity in a complete medium. *S. faecalis* filtrate enhanced the final toxicity when present in high concentrations in the presence of phosphate buffer.

Introduction

Despite the rarity of botulism (11), considerable evidence has accumulated which supports the ubiquitous nature of *Clostridium botulinum*. The possibility that accompanying organisms may influence botulinal development has been recognized. *C. botulinum* has been isolated infrequently from unsterile processed foods (10, 13), and even experimentally inoculated samples frequently fail to become toxic (13).

The effects of mixed cultures on the development of *C. botulinum* have been reported to include symbiosis (6, 12, 14), antagonism (6, 8), inhibition (7, 14), and, as well, the destruction of preformed toxin (7, 16). The potentiation of type E toxin by a concomitant *Clostridium* is also on record (15).

The earlier literature contains conflicting evidence, because adequate assay methods for toxin and much information on the differences in behavior between inocula of botulinal vegetative cells and spores were unavailable. Autolysis of the cells of *C. botulinum*, now recognized as the chief mechanism for release of the toxin (2, 9), was then not widely appreciated.

We felt that experiments designed to study the effect of the filtrates of representative concomitant bacteria on toxigenicity, spore germination, and cell lysis of *C. botulinum* might help to clarify the mixed-culture situation. This approach seemed feasible because most reported mixed-culture studies on *C. botulinum* have indicated the initial predominance of the aerobic coexisting types, or the common putrefactive anaerobe, either as they occur in nature, or when they are artificially inoculated together. Subsequent growth and toxigenicity of the toxic anaerobe might then greatly depend on any influence that may be exerted by the accompanying organism's metabolites and/or other substances resulting from its growth.

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This paper presents the results of initial studies of the effects, on *C. botulinum* type A, of the filtrates of four well-characterized concomitant types.

Materials and Methods

Clostridium botulinum type A, designated here as the Hall-FD strain, has been previously described (4). A nonsporulating strain, this culture was used in studies of toxigenicity and cell lysis. The sporulating strain, also type A, No. 7948, was obtained from the American Type Culture Collection, as was *Bacillus sphaericus* (*B. fusiformis*), No. 7055, and *Streptococcus faecalis*, No. 6057. *Escherichia coli*, No. 34, was received from the Midwest Culture Service, Terre Haute, Indiana. We are indebted to Dr. L. L. Campbell, Jr., State College of Washington, Pullman, Washington, for the culture of *Clostridium sporogenes* (P. A. 3679), which is the strain A of Dr. J. C. Ayres, Iowa State College, Ames, Iowa.

Details of maintenance of the two botulinus strains are described elsewhere (4). *C. sporogenes* was maintained in Cooked Meat Phytone Medium (BBL) containing twice the recommended amount of dry solid per volume of water. The three aerobes were kept in stab cultures in CTA Medium (BBL). All cultures of the concomitant bacteria were incubated for 18–24 hours at 35° C, stored in the refrigerator, and transferred biweekly.

Full details concerning preparation of media, filtrates, spore suspensions, washed cell suspensions, and the methods of testing toxicity of cultures, spore germination, and cell lysis have been described (3) and will be mentioned briefly only for clarity.

Both buffered and unbuffered versions of a complete medium (4) were employed, the unbuffered type being used for filtrate production, and the buffered form for toxigenicity tests and spore germination studies. However, in order to facilitate dilution of filtrates, and to prevent salting out of the organic components, the N-Z Amine B – autolyzed yeast extract solution and the phosphate buffer, pH 6.3–6.5, were made and autoclaved separately in quadruple strength. The two portions were combined as needed, and 40% (w/v) glucose solution (autoclaved separately) was added to give a glucose concentration of 1% in the double-strength medium. This medium was then added to equal volumes of sterile diluted filtrates or distilled water as needed to provide equivalent concentrations of fresh medium in all experiments. A spore production medium consisting of 5% Trypticase (BBL) and 0.5% Bacto-peptone (Difco) in distilled water was also employed.

Sterile filtrates of 96-hour mass cultures of the concomitant bacteria, grown at 35° C without agitation, were prepared by preliminary centrifugation at 2000 r.p.m. and Seitz filtration at room temperature. They were stored at −20° C and used within 3 days.

Spores of *C. botulinum*, No. 7948, were produced by growing this strain at 35° C for 15 days. The spores were harvested by centrifugation at room temperature at 2000 r.p.m. They were washed three times with sterile distilled water, resuspended by shaking with glass beads, and immersed in boiling water for 15 minutes to kill vegetative cells. Suspensions were immediately cooled and stored in the refrigerator until they were used.

Cell suspensions of *C. botulinum* Hall-FD were made by growing mass

cultures of the organism in single-strength buffered medium for 20 hours at 35° C. They were harvested by centrifugation at 2000 r.p.m. at room temperature, washed three times, resuspended in sterile 0.2 *M* phosphate buffer (pH 7.0), and used immediately.

Toxigenicity of *C. botulinum* Hall-FD in the presence of 1:2 dilutions of bacterial filtrates in single-strength media (one volume of filtrate added to one volume of inoculated double-strength medium) was determined in 96-hour cultures by employing a goldfish assay for the 50% lethal dose of botulinus toxicity (4). Similar determinations were made in the presence of 1:8 dilutions of *C. sporogenes*, *E. coli*, and *S. faecalis* filtrates and 1:4 *B. sphaericus* filtrates because previous experiments had indicated that growth at these filtrate levels was about comparable with that in single-strength medium alone (3).

The method of Treadwell *et al.* (18) was modified for studies of spore germination in 18-mm Thunberg tubes. Appropriate dilutions of bacterial filtrates and double-strength buffered medium were added to the main tubes. Spore suspensions were heated at 75° C for 20 minutes in a water bath and were then put into the side arms. After evacuation, temperature equilibration, and mixing, the reaction was followed turbidimetrically at an incubation temperature of 35° C.

Botulinal cell lysis in the presence of filtrate was determined in systems in which equal volumes of diluted filtrates or fresh unbuffered medium and cell suspensions in 0.2 *M* buffer were added to sterile, optically matched tubes to give a final 0.1 *M* buffer system. Systems containing diluted filtrates which were heated in boiling water for 15 minutes were also studied. Optical density measurements were made at intervals up to 96 hours, and, in addition, initial and final pH readings were taken.

All turbidimetry was carried out in the Coleman Junior Spectrophotometer, Model 6A, at 665 mμ.

Results and Discussion

Toxicity values recorded in Table I indicate that of all the dilutions of the various filtrates tested only that of *S. faecalis* in a 1:2 concentration exerted an influence on toxigenicity of the Hall-FD strain. A significant increase in LD_{50}, to 5.8, noted with this filtrate in the buffered medium, apparently was dependent on a relationship between high filtrate concentration and phosphate buffer. This was evident in the fact that 1:8 *S. faecalis* filtrate showed no influence, and that when the 1:2 unbuffered cultures adjusted to pH 6.0 were tested, no significant change in toxicity was noted. Both the adjusted unbuffered cultures and the cultures in buffered medium remained at pH 6.0 throughout the 4-day incubation period. This points up the likelihood that phosphate content and not buffering *per se* was important in producing the increased toxigenicity noted in the buffered cultures containing 1:2 *S. faecalis* filtrate.

It is problematical whether the increased toxicity noted in the botulinal cultures containing *S. faecalis* filtrate has any significance in nature. Further investigations are necessary on environments which may involve the requisite relatively high *S. faecalis* filtrate – phosphate situations together with growth

TABLE I

Toxicity of centrifugates of *C. botulinum* Hall-FD cultures in complete media containing bacterial filtrates

Culture medium	Additive	Goldfish LD_{50} for 0.05-ml dose of centrifugate of 96-hour culture containing various additive dilutions			
		0	1:2	1:4	1:8
Buffered	None	4.4*	—	—	—
	Fresh unbuffered medium	—	4.7	—	—
	B. sphaericus filtrate	—	4.7	4.3	—
	C. sporogenes filtrate	—	4.5	—	4.5
	E. coli filtrate	—	4.6	—	4.5
	S. faecalis filtrate	—	5.8	—	4.6
Unbuffered	*S. faecalis* filtrate	—	0†	—	—
	S. faecalis filtrate	—	4.5‡	—	—

*Indicates that 0.05 ml of a $10^{-4.4}$ dilution of culture centrifugate was fatal to 50% of the goldfish within 72 hours after administration of the toxic dose.
†No growth occurred. The initial pH (4.2) of this system was below the lower limit (pH 5.3) of the Hall-FD strain (1).
‡Initial pH was adjusted with sterile NaOH to pH 6.0, equivalent to a 1:2 dilution in the buffered medium.

conditions suitable for the toxic anaerobe.

Figure 1 illustrates the change in light transmittance, from an average initial value of 65% T, of spore suspensions in the presence of culture filtrates as compared with appropriate controls. Maximum changes occurred by approximately 18 hours. Similar experiments with 1:2 filtrate generally showed the same activity relative to the controls although germination rates were generally reduced. *E. coli* filtrates at the higher concentration definitely showed more inhibitory activity whereas at the 1:8 level their activity was negligible. The other three filtrates were inhibitory at both levels.

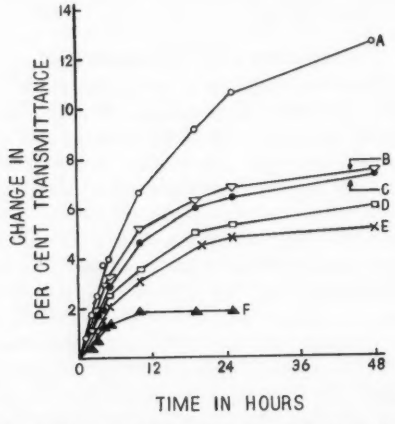

FIG. 1. Change in per cent transmittance of spore suspensions of *Clostridium botulinum* (ATCC 7948) in buffered complete medium containing 1:8 dilutions of bacterial filtrates. A, buffered medium alone; B, 1:8 fresh unbuffered medium added. Filtrates added: C, *E. coli;* D, *B. sphaericus;* E, *S. faecalis;* F, *C. sporogenes.*

The buffered control system containing no additives showed somewhat greater germination of spores before vegetative outgrowth than the system to which 1:8 fresh unbuffered medium was added. Such a result was not unexpected since it was the authors' general experience that buffered medium alone required a slightly greater vegetative inoculum for initiating growth than unbuffered medium alone or when added to buffered medium as a control. This seems to have been associated with the necessity for a greater accumulation of germinated spores before vegetative growth could be initiated, and does not appear to invalidate the observed inhibition of germination by the filtrates. The variety of the species of concomitants used suggest that germination inhibition may be widespread throughout natural environments containing the toxic anaerobe.

Figure 2 shows that lysis of botulinus cells was markedly increased by 1:2 dilutions of filtrates of *B. sphaericus* and *C. sporogenes*. Lysis was slightly decreased in the unbuffered-medium control and *E. coli* and *S. faecalis* filtrates, as well as heated filtrate systems, for which the four curves were practically superimposed. Comparable increases and decreases were found in systems containing 1:8 dilutions of filtrates except that the relative activities were much less. All cultures remained at neutrality during the 4-day incubation period except ones containing the 1:2 dilutions of *S. faecalis* filtrate, and they showed a final pH of 6.6, still within the accepted optimal range for autolysis (1).

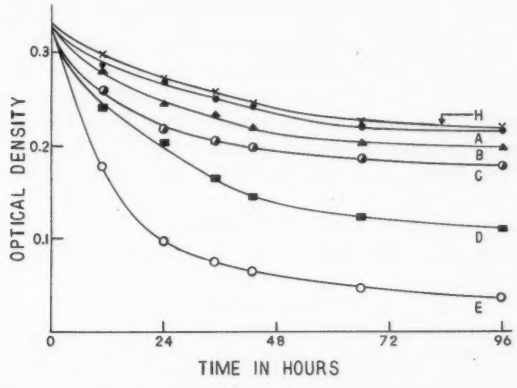

FIG. 2. Lysis of washed cell suspensions of *Clostridium botulinum* Hall-FD in 0.1 *M* phosphate buffer, pH 7.0, containing 1:2 dilutions of heated and unheated bacterial filtrates, and fresh unbuffered medium. A, *E. coli and S. faecalis* filtrates; B, fresh unbuffered medium; C, buffer alone; D, *C. sporogenes* filtrate; E, *B. sphaericus* filtrate; H, all four heated filtrates.

We feel that under the conditions used little significance should be attached to the lower lytic rates found in systems with added fresh unbuffered medium, unheated *E. coli*, and *S. faecalis* filtrates, as well as all four heated bacterial filtrates. It appears that their action was most likely a simple protective effect exerted by organic matter.

The lytic mechanism of *B. sphaericus* and *C. sporogenes* filtrates may be due to direct action by their lytic enzymes (suggested by its heat-labile character) or stimulation of botulinal autolysis by heat-labile components. The results also suggest the possibility that the lysis of cells of the toxic anaerobe by substances arising from growth of these concomitants may affect the initiation of growth of botulinal cells or germinated spores in mixed cultures.

The experimental results tend to call attention to some earlier observations on the putrefactive anaerobe. *C. sporogenes* spores inoculated into culture systems containing approximately equal numbers of botulinus spores have been shown to inhibit the development of toxicity of the latter organism (8). Subsequently, Dack (5) observed that botulinus spores were able to produce only scanty growth in filtrates of *C. sporogenes*.

C. sporogenes is commonly found in containers of food which have been inadequately heat-processed. We feel that the comparative rarity of toxic samples from batches of such food may be at least partly due to the spore-inhibitory, cell-lysis mechanism of substances produced by growth of the putrefactive anaerobe. We suggest that similar properties possessed by *B. sphaericus*, and perhaps related organisms as well, may contribute to the well-recognized difficulty in demonstrating toxicity in gross soil cultures unless they are first heated (7).

Inhibition of spore germination by filtrates of *E. coli* and *S. faecalis* indicates that the growth of these organisms may produce substances which make the intestinal tract unsuitable for botulinal development. Thom *et al.* (17) have reported the failure of development of toxemia in animals which had been fed toxin-free spores. Several subsequent recoveries of *C. botulinum* were made from the feces of the subjects. Delayed toxemia of animals in similar experiments has also been recorded (19). It is commonly accepted that botulism in a recognizable form does not develop from spores unavoidably ingested with fresh vegetables grown in contaminated soils.

Acknowledgment

The aid and counsel of Dr. Robert B. Yee is gratefully acknowledged.

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ARTIFICIAL CULTURE OF CLOSTRIDIUM BREVIFACIENS N.SP. AND C. MALACOSOMAE N.SP., THE CAUSES OF BRACHYTOSIS OF TENT CATERPILLARS[1]

GORDON E. BUCHER

Abstract

Clostridium brevifaciens n.sp. and *C. malacosomae* n.sp. were isolated and cultured from larvae of tent caterpillars suffering from brachytosis. Cultures of both species produce the disease when fed to susceptible larvae. The bacteria are described and directions are given for preparing the specialized medium in which they grow. *Clostridium brevifaciens* is the primary cause of brachytosis of *Malacosoma pluviale* (Dyar) in nature, but *C. malacosomae* sometimes occurs in mixed infections and, by itself, produces similar symptoms of disease in experimental larvae.

Introduction

Bucher (2) described a new disease of larvae of the tent caterpillar, *Malacosoma pluviale* (Dyar), caused by a sporeforming bacterium, but failed to isolate the bacterium in pure culture and consequently did not name it. Further investigations resulted in the isolation and culture of two bacteria associated with the disease. This paper names the disease, describes the causal bacteria and a medium on which they grow, and demonstrates that the cultured bacteria produce the disease in tent caterpillar larvae.

The name "brachytosis", from the Greek *brachys* (short) and *-osis* (a morbid condition), is proposed for this disease because the most striking symptom of diseased larvae is their shortening before death.

Preparation of Culture Media

After repeated failure to culture the bacteria on conventional bacteriological media (2), even when these were made alkaline and anaerobic, a successful culture medium was devised on the basis of four observations and hypotheses: the mid-gut of both healthy and diseased larvae is highly alkaline (2); bacteria are often arranged in a pincushion effect about leaf cells in the lumen of the gut (2), an observation suggesting that the cells provide some essential nutrient; the mid-gut of several insects provides anaerobic conditions (4) and that of the tent caterpillar is probably anaerobic; and a number of nutritionally fastidious bacteria are known to require a high ratio of potassium to sodium. The successful culture medium satisfied all four requirements simultaneously: it was highly alkaline and anaerobic, and contained an extract of leaves and a high concentration of potassium to sodium.

Preparation of the Leaf Extract

The following method of preparing leaf extract for supplementing a basal medium was finally adopted as standard because it consistently produced the best growth of the bacteria. The formula was: apple leaves, 50.0 g; ascorbic acid, 0.4 g; distilled water, 250.0 ml. Mature apple leaves were cut

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from any commercial variety of the domestic apple (*Malus pumila* Mill.), weighed after removal of the stems and midribs, and macerated with water in a home-type blender to a smooth mixture. The ascorbic acid prevented excessive oxidation and browning during blending. The maceration was squeezed through cheesecloth and the resulting liquid with fine suspended particles was chilled to $5 \pm 1°$ C in a cotton-stoppered flask; the pH was about 5.0. After chilling, 5 ml of ethylene oxide and 10 ml of normal potassium hydroxide (both chilled to 5° C) were added and the extract was refrigerated for 2 or 3 days; the pH was about 9.0. The extract was then brewed at room temperature for at least 2 weeks. As ethylene oxide is an effective cold-sterilizing agent (1), flasks of extract rarely became contaminated during the brewing period. Ethylene oxide is highly volatile at room temperature and thus rapidly escaped from the brew. KOH improves the efficiency of ethylene oxide and produces some precipitate, which is removable by filtration, thus reducing excessive cloudiness in the final medium. During brewing, much of the suspended particulate matter settled out. That which remained in suspension could not be economically removed by centrifuging or by filtering through Whatman No. 1 papers. Thus the supernatant was carefully removed and filtered through a Seitz filter. To ensure sterility, it was refiltered through a sterilized Seitz filter just before addition to the basal medium. The resulting fluid was dark brown, clear, had a pH of about 7.0, and hereafter is called "leaf brei".

Preparation of Other Ingredients

The basal medium was Tryptose phosphate broth prepared according to the directions of the manufacturer, Difco Laboratories, Detroit, Michigan. To each liter, 2.0 g agar was added to maintain anaerobic conditions and 0.01 g methylene blue may be added optionally to indicate the oxidation-reduction potential. The basal medium was dispensed in 100-ml amounts in 250-ml flasks and terminally sterilized in the autoclave. If stored it was reliquefied before use.

Approximately normal potassium hydroxide was made by dissolving 56.1 g of KOH pellets in a liter of freshly distilled water, tubing in 10-ml amounts in screw-capped tubes, and terminally sterilizing in the autoclave.

A 1.0% aqueous solution of cysteine hydrochloride and a 0.05% aqueous solution of thiamine hydrochloride were sterilized by Seitz filtration and tubed aseptically in 10-ml amounts. They should not be stored for more than 2 days.

Compounding the Medium

The formula for the final medium follows:

	Volume	% w/v of final medium
Tryptose phosphate broth	100 ml	
Bacto-tryptose		1.0
Glucose		0.1
Sodium chloride		0.25
Disodium phosphate		0.125
Agar		0.1
Methylene blue		0.0005
Cysteine hydrochloride	10 ml	0.05
Thiamine hydrochloride	10 ml	0.0025
Leaf brei	70 ml	
N potassium hydroxide	10 ml	0.28

The cysteine, thiamine, leaf brei, and KOH were added aseptically to the flask containing the warmed Tryptose phosphate basal medium. The addition of KOH produced a fine precipitate that remained in suspension and caused some cloudiness of the final medium, but less precipitate was produced when the KOH was added last. The pH of the medium was 10.2 ± 0.2. If below this range, the pH was adjusted with 1 or 2 ml of *N* KOH. The culture medium was tubed aseptically in 8-ml amounts and was ready for use after 2 or 3 days of incubation to verify its sterility.

This fluid medium was amber to reddish brown, and slightly cloudy. Neither the color nor the precipitate was sufficiently strong to prevent the recognition of bacterial growth by a visible increase in the cloudiness of the medium, but changes in color and density of the precipitate from batch to batch prevented the erection of standards that allowed growth to be estimated by a densitometer. Therefore, growth of the bacteria was followed by aseptically removing a drop and examining it microscopically under dark-field illumination.

The pH of the medium fell slowly but continuously, from 10.2 when tubed, to 9.8 ± 0.2 in 3 to 7 days and to 8.5–8.7 after several months of storage. Apparently the alkali combined slowly with substances in the leaf brei. Reaction of the alkali with CO_2 of the atmosphere did not account for the continuous fall of pH, as this fall also occurred when the medium was stored in sealed tubes.

The medium occupied the bottom 60 mm of a 15×150-mm culture tube. Throughout most of this volume it provided anaerobic conditions. A day or two after tubing, the top 4 or 5 mm became clear and slightly darker in color. This portion was aerobic and aerobic bacteria grew in this layer whereas anaerobic contaminants grew in the body of the medium. When methylene blue was added, the surface layer was much darker because of the blue color of the oxidized indicator. As the E_0[1] of methylene blue at pH 10 is -80 mv, the redox potential of the body of the culture medium, where methylene blue was colorless, was probably -140 mv or lower and the medium was strongly reducing. During storage the depth of the aerobic surface layer slowly increased to 10 mm after several months. Tubed media supported growth of the bacteria after storage at 5° C for several months.

A solid medium that supported colonial growth of the bacteria in plates was prepared by adding 50 g of agar, 4.0 g of glucose, and 4.0 g of sodium thioglycollate to 1 liter of Tryptose phosphate broth and sterilizing this basal medium in 100-ml amounts. When it had cooled to about 65° C, cysteine, thiamine, leaf brei, and KOH were added in the same proportion as in broth, the pH was adjusted to 10.2 ± 0.2, and eight thick plates were poured immediately. The agar was ready for inoculation as soon as its surface was dry. It did not support growth of the bacteria if stored for more than a day or two, probably because it was slowly oxidized when exposed to air. The redox potential of the agar could be estimated by adding 0.04 g methylene blue to each liter of the basal medium, but this concentration (0.002% w/v in final medium) slightly inhibited growth of the bacteria and is not recommended for routine plating. After inoculation the plates were incubated anaerobically under hydrogen in Brewer jars or with Brewer anaerobic covers.

Description of *Clostridium brevifaciens* n.sp.

In the Gut of the Host

Vegetative rods.—Gram-negative; occurring singly and rarely in pairs; highly variable in size because rods grow both in diameter and in length prior to sporulation, length of living rods under dark-field illumination about 6 to 7 μ (normal range 3 to 13 μ), width about 1.0 μ (normal range 0.9 to 1.3 μ); typically straight with parallel sides and rounded ends, optically empty without crystalline inclusions in dark-field or phase-contrast illumination; readily stained by bacterial stains, unevenly stained by Giemsa stain especially when beginning to sporulate; motile with a characteristic fishlike swimming motion, usually proceeding in one direction until stopped by an obstacle, flagellation unknown.

Sporulating rods.—Always single; length about 9 μ (normal range 7 to 14 μ), width about 1.7 μ (normal range 1.4 to 2.0 μ); sides parallel, ends rounded, without crystalline inclusions; unevenly stained by Giemsa; motile as vegetative rods; bearing oval subcentral or subterminal spores without bulging of the sporangia.

Spores.—Liberated by autolysis of mature sporangia; about 1.6 by 3 to 3.5 μ; readily stained by carbol fuchsin, resist alcohol decolorization especially when newly liberated.

Source.—The gut and faeces of *Malacosoma pluviale* (Dyar) suffering from brachytosis.

In Broth

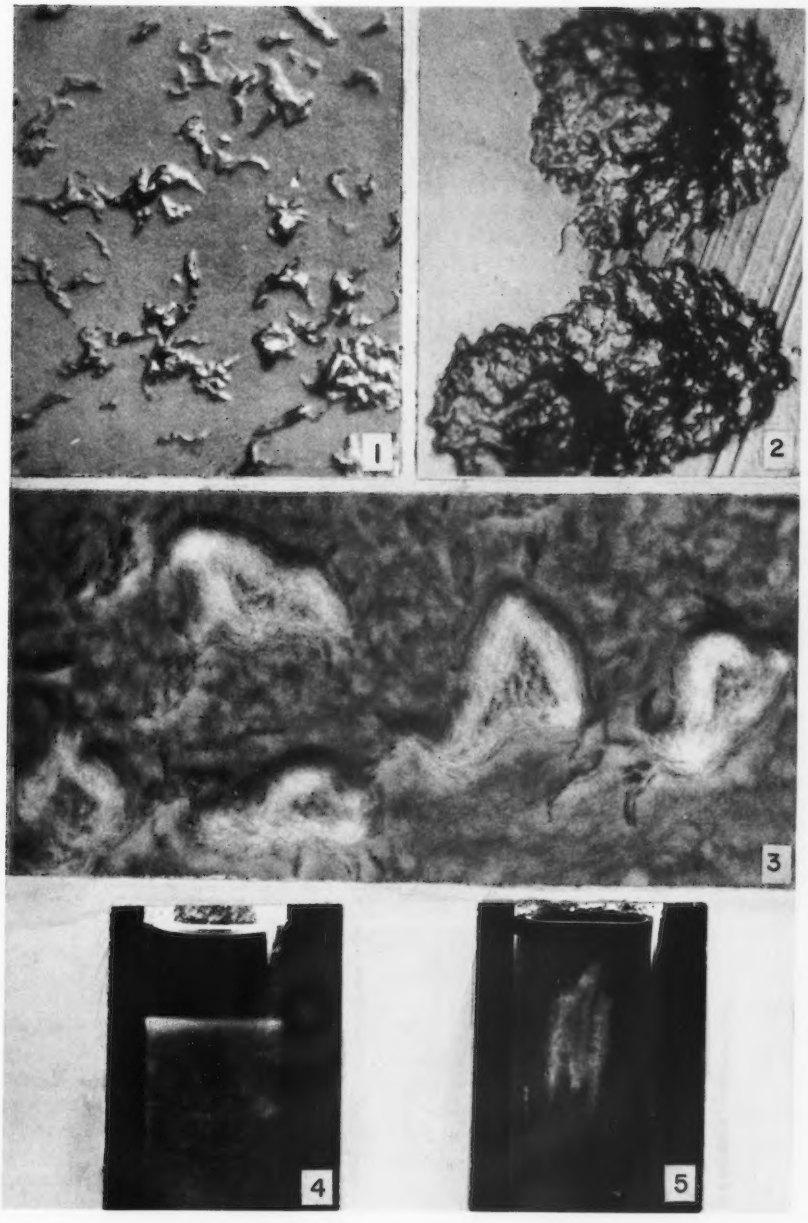
Vegetative rods.—Gram-negative; occurring singly, paired, or very rarely in chains of three; width about 1.0 μ, length highly variable depending on the medium and the speed of multiplication, usually 4 to 6 μ but may range from 3 to 20 μ; typically straight with parallel sides and rounded ends, optically empty in dark-field illumination; readily stained; motile with the same characteristic motion as rods in the host insect, flagellation unknown; do not sporulate.

Specific growth requirements.—Anaerobic conditions, high pH (8.5 to 10.2), high concentration of K to Na, some unknown components supplied by an alkaline extract of apple leaves.

Growth form.—A band of turbidity forms slightly below the junction of the aerobic surface layer and the anaerobic deeper layer and extends downwards into the medium for 2 to 4 cm, rarely to the bottom of the tube. In media that support heavy growth, the bacteria are concentrated into a narrow disk at the extreme top of this band of growth so that tubes viewed from the side show a sharp ring of heavy growth below which is a band with progressively decreasing turbidity and with a diffuse lower margin. The upper margin of the very dense ring or disk is sharply defined as the bacteria do not multiply above this zone and the medium remains clear (Fig. 4).

FIG. 1. Colonies of *C. brevifaciens*, 8 days old, photographed by reflected light. ×67.
FIG. 2. Colonies of *C. malacosomae*, 7 days old, photographed by reflected light. ×67.
FIG. 3. Photomicrograph of *C. brevifaciens* colonies by anoptral phase contrast illumination. ×670.
FIG. 4. Typical ring growth of *C. brevifaciens* in anaerobic area of broth.
FIG. 5. Typical veil growth of *C. malacosomae* in anaerobic area of broth.

PLATE I



Bucher—Can. J. Microbiol.

On Agar

Colonies.—Microscopic in size; greatest diameter 50 to 150 μ after 3 to 7 days of incubation; only one or two cells thick; appear as colorless, transparent, tiny flecks on the surface of the brown agar when viewed with a stereoscopic binocular microscope (Fig. 1); under the compound microscope colonies are usually elongate, up to three times as long as broad, margins irregular, often with wisps of cells protruding from the edge (Fig. 3).

Vegetative rods.—Tend to line up with their long axes parallel to the long axis of the colony; similar to those in broth culture; nonmotile when removed from plates and suspended in wet mounts, but sometimes motile in droplets of condensed water on the agar surface; do not sporulate.

Specific growth requirements.—Strict anaerobiosis as produced in Brewer jars under H_2 or in well-sealed Brewer plates with thioglycollate incorporated in the agar. Growth on agar is much poorer than in broth; only a small fraction of the rods of the inoculum produce colonies, the remainder lyse where deposited. Most colonies form at or near the periphery of the inoculation or grow out from the periphery into the uninoculated portion of agar. Colonies grow slowly, being recognizable in 3–4 days and increasing in size for 7–10 days of incubation.

Description of *Clostridium malacosomae* n.sp.

In the Gut of the Host

Vegetative rods.—Gram-negative; occurring singly or in pairs; variable in size, usually 4 to 7 μ long and about 1.0 μ wide; typically straight with parallel sides and rounded ends but longer rods sometimes bent or slightly twisted, optically empty in dark-field illumination; readily stained by bacterial stains, unevenly stained by Giemsa especially when beginning to sporulate; nonmotile.

Sporulating rods.—Usually single, sometimes paired; slightly fusiform, bearing oval, subcentral spores at the widest spot; length about 5 to 7 μ, width at the spore about 1.5 to 1.7 μ; without crystalline inclusions; unevenly stained by Giemsa; nonmotile.

Spores.—Liberated by autolysis of mature sporangia; slightly oval; about 1.5 by 2 to 3 μ; readily stained by carbol fuchsin.

Source.—The gut and faeces of *Malacosoma pluviale* (Dyar) suffering from brachytosis.

In Broth

Vegetative rods.—Gram-negative but sometimes retaining traces of blue dye when young; single, paired, or rarely in short chains; width about 1.0 μ, length variable but usually 4 to 7 μ; short rods with parallel sides and rounded ends, long rods tend to bend or twist sometimes abruptly; optically empty in dark-field illumination; readily stained; nonmotile; do not sporulate.

Specific growth requirements.—Anaerobic conditions, high pH, high concentration of K to Na, some unknown components supplied by an alkaline extract of apple leaves.

Growth form.—Growth extends downwards from the point of inoculation in fan-shaped or cone-shaped veils (Fig. 5) that may coalesce later. Growth does not progress above the point of inoculation so that the medium does not become uniformly turbid.

On Agar

Colonies.—Small, rarely exceeding 0.5 mm in diameter, almost invisible to the naked eye; under the stereoscopic binocular, colonies are roughly round, flat or very low convex, colorless, translucent, with irregular margins and rough or undulate glistening surfaces (Fig. 2); under the compound microscope, colonies appear to be composed of waved or coiled chains of rods, several cells in depth at the center and one cell thick at the periphery.

Vegetative rods.—Similar to those in broth culture but may be more bent or twisted; do not sporulate.

Specific growth requirements.—Strict anaerobiosis. Growth on agar is poorer than in broth but colonies develop in all portions of the inoculum. If the inoculum is heavy, growth may be coalescent and appear as a thin, colorless, translucent membrane. Colonies are recognizable in 2 or 3 days and grow for 7 to 10 days of incubation.

Reasons for Nomenclature

Though neither of the bacteria has been induced to sporulate in artificial culture, experiments, described below, demonstrated that pure broth cultures produced typical symptoms of brachytosis in susceptible larvae. Thus, regardless of their inability to sporulate in prevailing culture media, these bacteria must be regarded as sporeformers and placed in the genus *Clostridium*, which includes all of the sporulating bacteria that are obligate anaerobes.

These bacteria differ from known members of the genus in a number of factors, including their demands for specific nutritional factors and high pH, their large diameter, and their ability to multiply in the gut of tent caterpillars and produce characteristic symptoms. Therefore they are regarded as new species. The name *C. brevifaciens*, from the Latin *brevitas* (shortness) and *faciens* (producing), is proposed for the bacterium that is the prime cause of brachytosis of tent caterpillars. The name *C. malacosomae*, from the generic name of the host, is proposed for the bacterium that is sometimes associated with brachytosis in natural infections.

In addition to differences in form and in manner of growth described above, the bacteria differ greatly in motility and degree of nutritional fastidiousness. *Clostridium malacosomae* is never motile either in artificial culture or in the gut of the tent caterpillar. Both vegetative and sporulating rods of *C. brevifaciens* are typically motile, but may lose motility towards the end of the infection cycle in tent caterpillars (2) or the end of the growth cycle in broth tubes, and when exposed to nutritionally deficient culture media, low pH (below 7.5), or aerobic oxidation-reduction potentials. Motility may be lost by individual rods as they age or by the whole culture as a result of age or unfavorable conditions, and is usually regained during the logarithmic growth phase that follows transfer into a favorable medium. However, some strains were isolated in which motion was slow and few rods were motile even during active multiplication; such strains may become completely nonmotile after several transfers and fail to regain this property even when rapidly and serially subtransferred to new media. These nonmotile strains do not form the typical ring growth in broth but grow as a band with a spreading diffuse lower margin. In respect to other features of form and of colony formation,

these nonmotile strains resemble the motile strains of *C. brevifaciens* from which they arose and do not form veils in broth like *C. malacosomae.* Thus there is no evidence to suggest that *C. malacosomae* is merely a variety of *C. brevifaciens* that acquired a different form and manner of growth as an accompaniment to loss of motility.

Clostridium malacosomae is much less fastidious in its growth requirements than *C. brevifaciens;* it produces denser populations in broth and larger colonies on agar; many rods produce colonies when inoculated onto agar, whereas only a tiny percentage of the rods of *C. brevifaciens* can initiate colony formation on an agar surface. Media made from different lots of leaf brei vary considerably in ability to support growth of *C. brevifaciens*, but support heavy growth of *C. malacosomae.*

Primary Isolation and Maintenance of Strains

Attempts to culture the bacteria that cause brachytosis from spore-laden frass of infected tent caterpillar larvae failed. Spore suspensions purified by differential centrifugation, pasteurization, and chemical treatment still contained from 40 to 300 contaminants per 100,000 spores. Media inoculated with such suspensions were rapidly overgrown by the contaminants, none of which produced brachytosis when fed in large doses to larvae (2). Inoculation of suspensions, from which the contaminants had been diluted out, failed to initiate growth, and ungerminated spores were visible on the agar surface for 2 weeks. It appeared that few, if any, spores could germinate in either solid or liquid culture media.

About 60% of 200 attempts to culture *C. brevifaciens* were successful when the inocula were obtained from the regurgitated fluid or mid-gut contents of infected larvae and placed directly in leaf brei broth. Such inocula were calculated to contain at least 20,000 motile vegetative rods and came from larvae with heavy infections of vegetative rods, few of which were sporulating. Unsuccessful attempts were due partly to contamination but mostly to failure of any bacteria to grow. In these cases the inocula came from insects with light infections or with advanced infections in which sporulating bacteria predominated. Primary isolation and culture directly on leaf brei agar was less successful than in broth because only a small percentage of vegetative rods initiated colony formation, and the remaining rods and spores remained dormant on the agar surface.

Isolations made from naturally infected larvae of *M. pluviale* from British Columbia or from experimentally infected larvae fed spores extracted from frass usually resulted in pure cultures of *C. brevifaciens*, but a small proportion resulted in growth of both bacteria. Despite its greater speed and vigor of growth, *C. malacosomae* produced only from one to a dozen well-isolated veils in broth or colonies on agar when first isolated in mixed culture with *C. brevifaciens.* It was assumed that each veil or colony resulted from the growth of a single cell or single clone of bacteria. If this assumption is valid, it follows that *C. malacosomae* occurs in only some of the naturally infected larvae and then only in small numbers in proportion to *C. brevifaciens.* These observations, coupled with the fact that *C. malacosomae* was never isolated in pure culture from naturally infected larvae, lead to the conclusion that *C. brevifaciens* is

the prime cause of brachytosis but that *C. malacosomae* is a common though not abundant companion, and that mixed infections frequently occur. Neither bacterium was isolated from healthy larvae, the regurgitation of which was microscopically free of typical bacterial rods.

Though *Malacosoma americanum* (F.) is experimentally susceptible to brachytosis, there is no evidence that field populations are infected. Larvae examined from over 200 nests in the vicinity of Belleville and Kingston and from 60 nests from other localities in Ontario showed no external or microscopic signs of brachytosis, and all attempts to isolate the bacteria in culture failed.

Mixed cultures of the bacteria obtained by original isolation were readily purified by several broth-to-broth transfers or by plating on agar and selecting typical colonies for reinoculation into broth. Strains used experimentally were purified by at least two such plating cycles. Twelve strains of *C. brevifaciens* and three of *C. malacosomae* have survived from 20 to 200 successive transfers through broth or agar. Strain differences are minor, but consistent from transfer to transfer. Strains of *C. brevifaciens* differ in motility and in rod length. Strains of *C. malacosomae* differ in rod length and in the degree of chaining and twisting of rods.

When originally isolated, both bacteria grew slowly, but they grew more vigorously after several transfers and may be considered as conditioned or adapted to the culture medium. After conditioning, *C. brevifaciens* produces cloudiness in broth at 24° C in 2 days, ring formation a day later, and a density of 1×10^8 motile rods per ml within 5 days at the densest portion of the tube. With further incubation the rods lose motility and finally lyse. After 14 days, the broth contains numerous ghosts often with tiny cytoplasmic inclusions, much unorganized debris, and few motile rods. Colonies on agar are visible in 3 to 4 days and grow for 7 to 10 days after which lysis begins. *Clostridium malacosomae* follows a similar cycle of growth and lysis. Veils are visible in 1 to 2 days, grow in extent and density (5×10^9 rods per ml) for about 5 days, and then become more diffuse as the bacteria begin to lyse. Lysis is nearly complete after 14 days. Thus strains are routinely maintained by weekly transfer. Both bacteria survive short periods of cold (4 ± 2° C) if refrigerated at the peak of growth. Transfers made after 3 weeks of refrigeration are successful but after 4 weeks an increasing number fail to grow.

Both species produce acid in broth: *C. brevifaciens* lowers the pH by 1.6 ± 0.3 units in 5 to 7 days if heavy growth occurs, and *C. malacosomae* lowers it by 0.9 ± 0.3 unit. The reduction of pH serves as a rough index of the amount of growth. After rapid multiplication has ceased, the pH remains constant at the lower level or may rise by 0.2 unit during extensive lysis of the rods. In cultures of *C. brevifaciens*, the lowest recorded pH was 7.1 following heavy growth in broth with an initial pH of 8.5. The pH seldom falls below 7.6 to 7.4 for *C. brevifaciens* or below 8.1 to 7.9 for *C. malacosomae;* it is probable that multiplication ceases as these levels are approached and that lysis is initiated, at least in part, by unfavorably low pH.

Several tests were performed to determine the minimum size of the inoculum of conditioned cultures required to ensure growth. Minimum inocula of 2000 to 5000 rods of *C. brevifaciens* were required to initiate growth in broth if

they were taken from young cultures in the logarithmic phase of multiplication, and larger inocula were necessary if they were taken from older cultures containing higher proportions of presumably nonviable rods. Inocula 10 times as dense were required to produce colonial growth on agar, and it appeared that perhaps only one in a thousand rods was capable of initiating the formation of a colony. *Clostridium malacosomae* initiated growth from inocula of less than 100 rods and probably can produce a veil or colony from a clone of viable rods of any small size. Thus failure to culture *C. brevifaciens* from all diseased larvae was attributed to the inability of this species to initiate growth in the medium unless the inoculum was large. On the other hand, failure to isolate *C. malacosomae* from diseased larvae indicated its absence or its presence in very small numbers.

A third anaerobic bacterium was isolated infrequently from the gut of tent caterpillars. It produced dense white clumps in broth and abundant wet mucoid coalescent growth in agar. Rods were Gram-positive, nonmotile, 0.4 to 0.6 μ in diameter and 3 to 6 μ long, and formed long unbranched chains or threads, often 200 to 400 μ in length, that tangled into cottony masses. It produced abundant growth on a wide variety of liquid or solid media with or without leaf brei supplement. It caused no disease when fed to tent caterpillar larvae. It was not usually visible in smears of the gut contents, but may persist in small numbers for it was reisolated from experimentally fed larvae. It did not form spores, produced acid without gas in leaf brei broth reducing the pH by 1.0 ± 0.2 units, and was identified as a species of *Catenabacterium*.

Experimental Production of Brachytosis

Proof that *C. brevifaciens* and *C. malacosomae* cause brachytosis was obtained by feeding tent caterpillar larvae cultures of these bacteria.

Experimental larvae of *M. pluviale* were reared in the laboratory from egg masses collected in British Columbia; larvae of *M. americanum* and *M. disstria* Hbn. were collected in the field. Methods of rearing larvae and performing the tests were given previously (2, 3). The source of the larvae had no effect on the experiments as long as none of the larvae in the group were diseased. Some lots of *M. pluviale* reared from field-collected eggs developed brachytosis without treatment (3). More rarely, nests of *M. americanum* became infected after several days in the laboratory, presumably by contamination with air-borne spores. Diseased material was not used.

Each test was performed on larvae selected from a single nest or reared from a single egg mass. The population was scanned for obvious symptoms of disease and several larvae at random were examined microscopically for the presence of virus polyhedra in the tissues and for the characteristic rods of brachytosis in the lumen of the gut. If the population was apparently healthy, the larvae were divided into several equal groups of a single instar. One group was picked at random as a control, the others were fed various cultures and doses of bacteria. Observations were continued until all the larvae had died or formed cocoons. In some tests observations were also made on the ability of prepupae to pupate and of pupae to produce normal adults. Observations consisted of recording gross symptoms of brachytosis (2) and mortality from brachytosis or other causes, and periodic microscopic examination of

the regurgitation of sample larvae for the presence of typical bacteria. The diagnosis was sometimes confirmed by reisolating the specific bacterium in artificial culture. An experiment was considered valid if no macroscopic or microscopic signs of infection appeared in the control group. In some experiments the control group did show symptoms of brachytosis either because of contamination during a long experiment or because of light, nondiagnosable infection in the original population. Such experiments were considered invalid and the results discarded. Demonstration of numerous typical vegetative and sporulating rods of the bacteria in the regurgitation or gut of test insects was accepted as evidence that the culture had produced the disease regardless of the later development of gross symptoms or the extent of larval mortality.

Tests were of two types. In the group test, larvae were fed as a group on leaves smeared with a known dose and the group was reared together as a unit; the dose was expressed as the mean number of bacteria per larva though obviously some individual larvae consumed more bacteria than others; moreover, throughout the test the larvae were continually exposed to reinfection if any individuals developed brachytosis from the original dose. In the individual test, each larva of the group was individually reared in a vial and fed a known dose of bacteria on a small segment of leaf; thus every larva of the group received the same dose within the limits of experimental error and avoided reinfection from other larvae.

Doses were estimated by using known volumes of cultures or dilutions, the density of which had been determined by visual counts of the bacteria in a counting chamber. The accuracy in terms of the number of vegetative rods applied was within $\pm 30\%$, but no true estimates could be made of the number of viable rods consumed by larvae. In any culture only a small proportion of the rods may be capable of growth and multiplication in the insect gut—i.e., in an infective state. Viability is rapidly lost on exposure to air through oxidation, desiccation, and change in pH, and infectivity is probably affected to a greater extent. Experimental larvae feed at different rates and thus the dose of infective rods consumed by any given larva may bear no constant ratio to the dose offered to it. Therefore the ingested doses of infective rods are unknown and probably are only minute and inconstant fractions of the doses applied.

The degree of infection caused by feeding cultures of *C. brevifaciens* and *C. malacosomae* to larvae of tent caterpillars of different ages and species is shown in Tables I to V. A number of conclusions were drawn from both the data of the tables and the accompanying observations:

(*a*) Cultures of *C. brevifaciens* produce brachytosis when fed to tent caterpillar larvae. Experimentally infected larvae carry large numbers of motile vegetative and sporulating rods in the gut and regurgitation, and show the same symptoms of disease as larvae that are naturally infected or infected by spore suspensions. The bacterium can be recultured from infected larvae.

(*b*) Infection does not always result in death. Most larvae infected in instar II die, but resistance to the lethal effects of the disease increases with the age of the larvae. Fifth instar larvae less than 5 or 6 days from spinning are able to spin cocoons though the bacterium grows readily in the gut. Depending on

TABLE I

Occurrence of brachytosis in groups of *Malacosoma pluviale* larvae fed cultures of *C. brevifaciens* and *C. malacosomae*

Instar	No. of larvae	Bacterial culture[a]	Dose[b]	Mortality,[c] % +	Mortality,[c] % −	Infection,[d] %	Incubation period,[e] days A	Incubation period,[e] days B
III	10	*C. brevifaciens* Bt_{39}	2×10^6	90	10	90 R	9	14
	10	Control		0	10	0		
III	30	*C. brevifaciens* Bt_{39}	5×10^5	73	10	81	10	15
	30	Control		0	7	0		
III	21	*C. brevifaciens* Dt_7	2×10^5	100	0	100	4	9
	21	*C. brevifaciens* Et_3	5×10^4	90	10	90	6	13
	21	*C. malacosomae* Bt_7	2×10^5 L	0	10	0	—	—
	21	Control		0	25	0		

NOTE: R, specific bacterium reisolated from infected insects in pure culture; L, culture old, rods beginning to lyse when fed.

[a]Letters B, D, etc. refer to specific bacterial strains; t_2, t_7, etc. refer to the number of consecutive transfers on artificial media.

[b]Dose is the estimated number of rods per larva offered to the insects and not necessarily the actual number of viable rods ingested.

[c]Mortality + indicates death of larvae with typical symptoms of brachytosis. Mortality − includes death of larvae from all other causes. Percentage of larvae that formed cocoons = 100 less total mortality.

[d]Infection indicates the percentage of larvae with microscopically visible, typical bacterial rods in regurgitation.

[e]Incubation period A is the elapsed time in days before infection was diagnosed by external symptoms or by the presence of typical bacteria in the regurgitation; B is the elapsed time before death of the first larva.

TABLE II

Occurrence of brachytosis in *Malacosoma pluviale* larvae individually reared and fed cultures of *C. brevifaciens* and *C. malacosomae*

Instar	No. of larvae	Bacterial culture[a]	Dose[b]	Mortality,[c] % +	Mortality,[c] % −	Infection,[d] %	Incubation period,[e] days A	Incubation period,[e] days B
II	7	*C. brevifaciens* At_{31}	2×10^5	*f*		70	4	11
	7	*C. brevifaciens* Bt_{31}	1×10^6	*f*		100	4	8
	7	*C. brevifaciens* Dt_{18}	3×10^5	*f*		100	4	8
	7	*C. brevifaciens* Et_{14}	6×10^5	*f*		100	4	8
	7	*C. malacosomae* Bt_{19}	1×10^6	*f*		57	4	7
	7	Control		0	29	0		
III	5	*C. brevifaciens* Bt_{41}	1×10^6 L	0	0	0	—	—
	5	*C. brevifaciens* Bt_{42}	2×10^4	80	0	100 R	7	9
	5	*C. brevifaciens*[g] A_rt_3	1×10^6	60	0	80 R	7	9
	5	*C. malacosomae* At_2	1×10^6	60	0	60 R	4	8
	5	Control		0	0	0		
IV	6	*C. brevifaciens* Bt_{42}	1×10^6	17	0	17	9	13
	6	*C. brevifaciens*[g] A_rt_3	1×10^6	33	17	50	8	13
	10	Control		0	0	0		
V	10	*C. brevifaciens* At_2	1×10^6	40	0	60 R	7	9
	10	Control		0	0	0		
V	20	*C. brevifaciens* Bt_{45}	3×10^5	45	0	55	5	10
	25	*C. malacosomae* Bt_2	1×10^6	64	0	72	4	10
	15	Control		0	7	0		
V Mature	16	*C. brevifaciens*[h] Bt_{45}	1×10^5	12	0	12 R	4	14
		C. malacosomae[h] Bt_2	2×10^5					
	16	Control		0	0	0		

[a, b, c, d, e] R, L—Same as in Table I.

[f]True mortality figures unavailable as some infected insects were fixed and sectioned, but observations indicated that all infected larvae would have died.

[g]Subscript on strain A_r indicates that culture was reisolated from larvae experimentally fed strain A.

[h]These cultures were fed in a mixture.

the severity of infection and the age of the larva when infection occurred, the larva within its cocoon may die as a larva, prepupa, or pupa, or emerge as a malformed or apparently perfect adult. Similar observations were made on larvae experimentally infected with suspensions of spores.

TABLE III

Occurrence of brachytosis in *Malacosoma pluviale* larvae individually reared and fed graded doses of *C. brevifaciens*

Instar	No. of larvae	Bacterial culture[a]	Dose[b]	Mortality,[e] % +	Mortality,[e] % −	Infection,[d] %	Incubation period,[e] days A	Incubation period,[e] days B
IV	20	*C. brevifaciens* Bt_{44}	2×10^6	5	5	40	5	16
	20	*C. brevifaciens* Bt_{44}	2×10^5	5	5	35	6	17
	20	*C. brevifaciens* Bt_{44}	2×10^4	10	25	35	8	20
	20	*C. brevifaciens* Bt_{44}	2×10^3	0	5	0	—	—
	20	Control		0	10	0		
IV	20	*C. brevifaciens*[g] B_rt_2	1×10^6	30	15	65	5	12
	20	*C. brevifaciens* B_rt_2	1×10^5	40	10	65	10	13
	20	*C. brevifaciens* B_rt_2	1×10^4	15	5	90	13	18
	20	*C. brevifaciens* B_rt_2	1×10^3	0	10	0	—	—
	20	Control		0	10	0		

[a, b, c, d, e, g] Same as in Tables I and II.

TABLE IV

Occurrence of brachytosis in groups of *Malacosoma americanum* larvae fed cultures of *C. brevifaciens* and *C. malacosomae*

Instar	No. of larvae	Bacterial culture[a]	Dose[b]	Mortality,[e] % +	Mortality,[e] % −	Infection,[d] %	Incubation period,[e] days A	Incubation period,[e] days B
IV	22	*C. brevifaciens* Bt_{44}	1×10^6	23	18	59	6	15
	22	*C. brevifaciens* Dt_{10}	1×10^6	9	9	50 R	4	17
	22	*C. brevifaciens* Et_8	3×10^6	9	14	27	6	11
	22	*C. malacosomae* Bt_{10}	1×10^6	9	9	55	11	12
	22	*C. brevifaciens*[f] spores	3×10^6	23	36	64 R	4	13
	22	*C. malacosomae*[f] spores	8×10^5	5	18	14 R	4	17
	22	Control		0	14	0		
V	20	*C. brevifaciens* Bt_{33}	1×10^6	0	0	30	4	—
	20	Control		0	0	0		
III	20	*C. brevifaciens* At_{172}	1×10^6	0	10	0	—	—
	20	*C. brevifaciens* Bt_{172}	1×10^6	0	15	0	—	—
	20	*C. malacosomae* Bt_{122}	1×10^6	0	5	0	—	—
	20	Control		0	10	0		
IV	20	*C. brevifaciens* At_{172}	1×10^6	0	5	0	—	—
	20	*C. brevifaciens* Bt_{172}	1×10^6	0	10	0	—	—
	20	*C. malacosomae* Bt_{122}	1×10^6	0	10	0	—	—
	20	*C. malacosomae* Ct_{46}	1×10^6	0	5	0	—	—
	20	Control		0	5	0		

[a, b, c, d, e, R—Same as in Table I.
[f]Spores obtained from frass of larvae experimentally infected by feeding cultures of the specific bacteria.

(*c*) There is little evidence for the dependence of infection or mortality on the offered dose except that the dose must exceed a certain minimum.

(*d*) There is an incubation period (A in tables) of several days between feeding and the multiplication of a population of bacteria in the gut of sufficient magnitude to enable positive diagnosis to be made by examining the regurgitation. This period increases with decreasing dose (Table III), and any factor that tends to reduce the true dose of viable and infective rods lengthens

TABLE V

Occurrence of brachytosis in groups of *Malacosoma disstria* larvae fed cultures of *C. brevifaciens*

Instar	No. of larvae	Bacterial culture[a]	Dose[b]	Mortality,[e] % +	Mortality,[e] % −	Infection,[d] %	Incubation period,[e] days A	Incubation period,[e] days B
III	15	*C. brevifaciens* At$_2$	6×10^5	13	47	67	5	9
	15	Control		0	53	0		
IV	35	*C. brevifaciens* At$_2$	3×10^5	0	6	60	5	—
	35	Control		0	6	0		
IV	15	*C. brevifaciens* At$_2$	3×10^5	7	7	40	6	13
	15	Control		0	7	0		
V	20	*C. brevifaciens* At$_2$	6×10^5	0	0	0	—	—
	20	Control		0	0	0		

[a, b, c, d, e] Same as in Table I.

the incubation period. Thus strains that have presumably lost some infectivity through numerous transfers tend to have longer periods than newly isolated strains. Infectivity is lost after repeated transfers (Table IV). The age of the larvae also affects the incubation period, which is shorter in young (instar II) and old (instar V) larvae than in those of medium age (Table II).

(*e*) The period (B in tables) between infection and death from disease is highly variable but tends to increase with decreasing doses or decreasing infectivity and with increasing larval age.

(*f*) Cultures that have passed the peak of multiplication and are beginning to lyse have little or no infectivity (Tables I, II).

(*g*) *Malacosoma pluviale*, the original host, is more susceptible both to infection and to the lethal effects of the disease than *M. americanum* or *M. disstria*. The latter is particularly resistant to the lethal action and most infected larvae survive.

(*h*) *Clostridium malacosomae* also produces brachytosis when fed to tent caterpillar larvae. Though the causal organism is different, it seems best to call the disease by the same name as the characteristic symptoms of the host are similar and as both bacteria may occur together in naturally infected insects. However, experimentally infected larvae carry large numbers of nonmotile rods in the gut and regurgitation, and reisolations from infected insects result in pure cultures of *C. malacosomae*. Other differences were observed. For example, the latent periods of the infection are usually shorter and this is coupled with a difference in the sporulating behavior of the rods. In the gut of the host, rods of *C. malacosomae* tend to sporulate rapidly and simultaneously and to lyse rapidly liberating free spores from the sporangia. As a result the insect gut contains either vegetative rods or free spores and infrequently contains numerous sporulating rods. In contrast, *C. brevifaciens* sporulates in more random fashion and the spores may be retained for long periods within motile sporangia; thus, throughout much of the infected period, the gut contains a mixture of rods in different stages of development. Such a mixture of stages was also observed in naturally infected insects (2).

(*i*) A mixture of cultures of *C. brevifaciens* and *C. malacosomae* produced infection, and both species were reisolated in culture from infected larvae.

Discussion and Conclusions

Clostridium brevifaciens and *C. malacosomae* have been commonly isolated in pure culture from tent caterpillar larvae suffering from brachytosis of natural origin or experimentally induced by feeding spore suspensions. Cultures of both bacteria produce gut infections and symptoms of brachytosis when fed to healthy larvae under conditions wherein control larvae from the same web remain uninfected. Therefore these bacteria are considered the cause of brachytosis despite some anomalies and weak links in the chain of evidence:

(*a*) The bacteria were not isolated in culture from all cases of brachytosis. This failure is due to the inadequacy of the culture medium. Spores do not germinate, many rods fail to grow, and large inocula are required to initiate growth in culture even after the strain has been conditioned by several transfers. Thus isolation occurs only from insects at the height of infection that carry huge numbers of dividing rods in the gut.

(*b*) Cultures produce a relatively low incidence of infection when fed to susceptible larvae and there is not a true regression between dose and percentage infection. This is probably due partly to inability to measure the true ingested dose and partly to great variation in susceptibility between larvae.

(*c*) Until the bacteria can be induced to sporulate in culture it is impossible to complete the chain of evidence for cause and effect by feeding spores harvested from artificial media. A similar situation occurs with *Bacillus popilliae* Dutky, which does not sporulate in culture media.

The conclusion that cultures of the bacteria are vegetative stages of the sporulating rods present in cases of brachytosis is supported by the observations that: (*a*) The bacteria have never been cultured from healthy insects. (*b*) When cultures of *C. brevifaciens* are fed, the gut becomes infected with numerous motile vegetative and motile sporulating bacteria of characteristic appearance and reisolation results in pure cultures of *C. brevifaciens*. (*c*) When cultures of *C. malacosomae* are fed, nonmotile vegetative rods develop in the gut and sporulating rods are also nonmotile and sometimes paired; only *C. malacosomae* can be reisolated. (*d*) Mixed cultures produce mixed infections and both species can be reisolated.

Clostridium brevifaciens is the primary cause of brachytosis in natural infections or in those induced by spores extracted from the frass of naturally infected larvae. Smears of the gut contents show the highly motile vegetative and sporulating rods typical of this species and very rarely show nonmotile, paired, slightly fusiform, sporulating rods that could be diagnosed as *C. malacosomae*. Despite the low inoculum required and the relative ease with which *C. malacosomae* grows in culture, this species has not been isolated free of *C. brevifaciens* though the latter is often isolated in pure culture. It is reasonable to conclude that most naturally diseased insects contain only *C. brevifaciens* and that the remainder contain only relatively small numbers of *C. malacosomae*. There is no evidence that mixed infections have any special significance for transmission or survival of the bacteria.

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AMIDE METABOLISM IN YEAST

III. ALIPHATIC AMIDE METABOLISM IN CANDIDA UTILIS[1]

A. DOMNAS

Abstract

The partial purification and the properties of propionamide, valeramide, and hexanamide deamidases are described. The purified deamidases hydrolyzed propionamide, valeramide, and hexanamide to form ammonia and corresponding fatty acids. Maximum enzyme activity was obtained at 37° C over a pH range of 6.7 to 7.8. The enzymes were inactivated at 55° C in 10 minutes. Propionamide deamidase, the most potent deamidase, was inhibited by ethylenediamine tetraacetate, arsenite, and cyanide, and was partially inhibited by iodoacetamide.

Evidence is presented which indicates the presence of more than one deamidase. Cell-free extracts hydrolyzed formamide, propionamide, valeramide, hexanamide. Lyophilized cells hydrolyzed acetamide, *n*- and iso-butyramide, isovaleramide, and glycinamide. A procedure is described for separating propionamide deamidase from valeramide and hexanamide deamidases.

Introduction

In 1936, Gorr and Wagner (2, 3) showed that a strain of *Torula utilis*, now classified as *Candida utilis* (Henneberg) Lodder and Kreger-van Rij, was able to grow on various amides such as acetamide, propionamide, and lactamide. The same investigators demonstrated the presence of deamidases for the previously mentioned compounds in dried cell preparations of the same yeast obtained from cultures grown on aliphatic amides. Recent work by Steiner (6) has shown that this organism is able to grow well on formamide, but that growth is poor with butyramide and valeramide. Steiner (6) has also demonstrated that dried cell preparations obtained from yeast which had been cultured on formamide, butyramide, or valeramide contained enzymes capable of deamidating these amides.

Since Steiner's work, no further investigation has been reported concerning the metabolism of aliphatic amides in yeast, nor any description concerning the nature of the enzyme(s) involved. This report is concerned with the partial purification of aliphatic amide deamidases and a description of some of their properties. Evidence is presented that indicates a specific enzyme for each amide substrate.

Materials and Methods

Organisms

The yeast *C. utilis*, ATCC No. 9950, was selected for this study. Stock cultures were maintained by monthly transfer on Difco yeast extract (0.5%) agar slants.

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Contribution from the Department of Biochemistry, Indiana University Medical Center, Indianapolis, Indiana. These studies were aided by a grant from the American Cancer Society P 178A. A portion of this work was done during tenure of a postdoctoral fellowship from the Arthritis and Rheumatism Foundation, New York.

Materials

Formamide,[2] acetamide, propionamide, *n*-butyramide, isobutyramide, *n*-valeramide, isovaleramide, and hexanamide were purchased from the Eastman Organic Chemicals. Glycinamide was obtained from Nutritional Biochemical Corporation.

Methods

The production of ammonia was used as the criterion of enzyme activity. Two methods of measurement were employed: (*a*) direct nesslerization and (*b*) microdistillation, using Conway microdiffusion vessels. For convenience, method (*b*) is termed the indirect method. Ammonia was determined by nesslerization according to the procedure of Koch and McMeekin (4) for both direct and indirect methods. Color development was measured with the Klett–Summerson colorimeter using the No. 54 filter. The indirect method was used as a check on the direct procedure and employed whenever interference by substrates or other materials was observed with Nessler's reagent. Protein was measured by the method of Lowry *et al.* (5).

Stability of Substrates

All aliphatic amides were tested for possible reaction with Nessler's reagents. Formamide and glycinamide gave lemon yellow colors, so that the direct method for these compounds was not usually employed. Incubation at 37° C of the various amides in 0.1 *M* phosphate buffer, pH 7.0, for 18 hours revealed no ammonia by either (*a*) direct or (*b*) indirect procedures. When the substrates were incubated for 40 hours with the salt and sugar medium which was used for the growth of the yeast, negligible amounts to no ammonia were found with all substrates with the exception of formamide. For enzyme assays, acetamide and propionamide were prepared fresh daily, because of the small amounts of ammonia which were formed on prolonged standing even at 4° C. (Glycinamide decomposed in alkali or when heated.) Our tests of amide stability agreed with those of Bray *et al.* (1).

Enzyme Assay

Deamidase activity was routinely measured in reaction mixtures containing 1.0 ml of extract, 1.0 ml of 0.1 *M* amide, and 1.0 ml of 0.1 *M* phosphate buffer, pH 7.1, in a total volume of 3 ml. The reaction mixture was kept in a water bath maintained at 37° C. At regular intervals 0.5-ml samples were removed and the protein precipitated by 0.05 ml of ice-cold 70% perchloric acid. The precipitated protein was removed by centrifugation and the supernatant liquid analyzed for ammonia. One unit of enzyme is defined as that amount of protein which forms 1 μM of ammonia from the aliphatic amide per hour at 37° C. Specific activity is expressed as micromoles ammonia formed per milligram protein per hour.

Effect of Enzyme and Substrate Concentration on Deamidase Activity

The release of ammonia from propionamide was found to be linearly related to the enzyme concentration. Figure 1 shows the data obtained with propionamide deamidase.

[2]Abbreviated in the tables as: form., acet., prop., *n*-but., isobut., *n*-val., isoval., hex., gly.

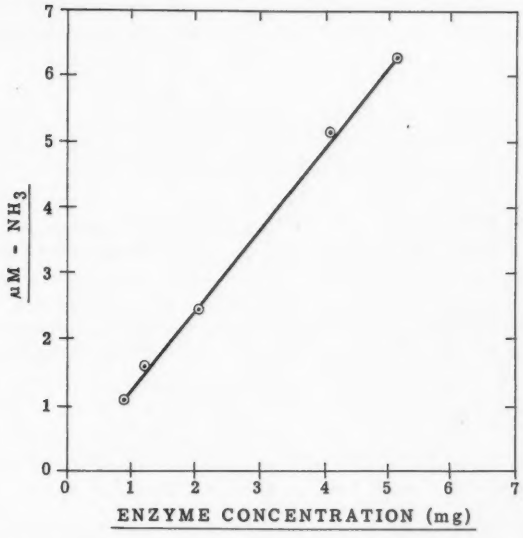

FIG. 1. Effect of enzyme concentration on propionamide deamidase activity of *C. utilis*. Enzyme assay as described in the text.

Enzyme Production

The medium used for enzyme production had the following composition: glucose, 20 g; KH_2PO_4, 0.5 g; $MgSO_4$, 0.2 g; amide equivalent to 0.5 g of nitrogen (usually acetamide); and distilled water to 1 liter. The amides were sterilized by filtration. Flasks containing 300 ml of the medium were inoculated with 1 ml of the 24-hour culture of the organism under study. The flasks were incubated at room temperature for 40 hours, and constant aeration provided by rotary shaking. The cells were harvested by centrifugation and washed twice with cold distilled water.

Dried Cell Extract Preparations

Dried cell preparations were made by lyophilizing whole cells from the frozen state at dry ice temperatures. Lyophilized cells were stored at −20° C until used for testing.

Purification of Aliphatic Amide Deamidases (Table I)

A. Crude Cell-Free Extract

To every 4 g of yeast cells (wet weight) suspended in 10 ml of 0.1 *M* phosphate buffer, pH 7.10, was added 10 g of glass beads (Fisher No. 11–312, 3 mm diameter). The cells were broken by shaking them at a rate of 320–330 oscillations per minute for 1 hour at 4° C. The mixture was centrifuged at low speed and the supernatant retained. The debris and unbroken cells were resuspended in the same amount of buffer as originally used above, and shaken for another hour. The supernatants of the first and second breakages were combined,

TABLE I

Purification of acyl deamidases of *Candida utilis*

Fraction	Units* with substrate:				Specific activity† with substrate:				$E\left(\frac{280}{260}\right)$
	Form.‡	Prop.	Val.	Hex.	Form.‡	Prop.	Val.	Hex.	
Crude cell extract	358	474	297	376	1.32	1.74	1.06	1.38	0.62
First protamine sulphate	217	251	158	234	1.97	2.28	1.44	2.13	1.0
Second protamine sulphate and phosphate buffer extraction	128	250	85.7	52.5	10.6	21.0	7.13	4.35	1.5

*One unit of enzyme is defined as that amount of protein which forms 1.0 μM of ammonia from the amide per hour at 37° C.
†Specific activity is defined as micromoles of ammonia released per milligram protein per hour.
‡Assayed by indirect method only.

TABLE II

% Hydrolysis* of aliphatic amides by extracts of *Candida utilis* grown on different amide sources

Nitrogen source	Substrate:								
	Form.†	Acet.	Prop.	But.	Isobut.	Val.	Isoval.	Hex.	Gly.‡
Acetamide	79.1	1.2	68.4	1.8	12.4	67.5	4.0	67.5	0
Propionamide	68.1	Trace	24.3	4.5	0	9	0	11.3	0
Butyramide	NT§	Trace	22.1	4.3	7.7	10.3	0	13.0	0
Isobutyramide	NT	Trace	40.7	6.0	13.2	16.7	3.9	23.5	0
Valeramide	5.8	0	29.7	3.4	0	10.2	0	14.3	0
Hexanamide	Trace	Trace	38.1	6.3	0	10.3	0	15.7	0
Glycinamide	NT	17.2	51.5	1.3	0	21.6	3.6	29.9	0
Urea	NT	Trace	16.7	8.1	0	6.3	0	7.2	0
Yeast extract	0	0	5.4	0	NT	0	NT	2.7	NT

NOTE: Reaction mixtures contained 1 ml cell-free extract adjusted to contain 2 mg protein per ml, 1 ml of 0.1 *M* PO_4 buffer, pH 7.0, and 1 ml of *M*/10 amide. Reactions were run for 18 hours at 37° C. Ammonia was determined by the direct and indirect procedures as described in the text.
*% hydrolysis is defined as (ammonia produced)/(available amide ammonia) ×100.
†Determined by indirect method only.
‡Determined by direct method only, because of excessive decomposition by alkalinization.
§NT: not tested.

and dialyzed for 12 hours at 4° C against several changes of cold distilled water. The extract was frozen overnight, thawed, and centrifuged to remove any insoluble matter which came down.

B. First Protamine Sulphate Step

One milligram of protamine sulphate in H_2O was added for every 22 mg of protein in the extract. The extract was allowed to stand for 2 hours at 4° C, and then centrifuged at 19,500 *g* for 30 minutes. The supernatant contained the deamidase activity.

C. Second Protamine Sulphate Step

Two milligrams of protamine sulphate dissolved in water was added for every milligram of protein in the extract. The mixture was allowed to stand for 12 hours at 4° C. The precipitate which formed was collected by centrifugation at 19,500 *g* for 30 minutes. The pellet was ground in a glass homogenizer with 15 ml of 1.0 *M* PO_4, pH 7.05, buffer for every 100 ml of step B extract. The water-clear supernatant, obtained following centrifugation of the protamine sulphate pellet suspension at 19,500 *g* for 15 minutes, contained the bulk of the deamidase activity.

Further attempts to purify the deamidases by different procedures such as sodium sulphate, acetone, and ethanol precipitations or calcium phosphate gels failed.

Deamidation of Aliphatic Amides by Crude Cell Extracts

Crude cell-free extracts of *C. utilis*, grown on various amide substrates, were capable of deamidating formamide, propionamide, butyramide, isobutyramide, valeramide, and hexanamide. As can be seen from Table II, cross-testing of the extracts against different amide substrates showed an interesting relationship. Regardless of the amide upon which the organism had been grown, deamidases for other amides were always found. The most active deamidase, always present, was propionamide, followed by hexanamide. However, these enzymes were weak in cells grown in yeast extract.

It was also observed that cell-free extracts contained little or no acetamidase, isovaleramidase, or glycinamidase acitivity, even in extracts from cells grown on the various substrates in question. No aliphatic amide or glycinamide deamidase activities were found in extracts of asparagine-grown yeast. It is of interest that the figure of 68.4% hydrolysis for propionamide agrees very closely with that of Gorr and Wagner (2).

Deamidation of Aliphatic Amides by Lyophilized Whole Cell Extracts

Whole cell extracts of yeast grown on acetamide or yeast extract deamidated acetamide, butyramide, isobutyramide, isovaleramide, and glycinamide (Table III). The enzymes deamidating glycinamide were found to be the most active in extracts of cells grown on yeast extracts. Isobutyramide and isovaleramide were also extensively hydrolyzed by yeast extract preparations. In contrast to this, acetamide-grown cells possessed a strong isovaleramide-deamidating activity, even though this substrate did not support yeast growth. The presence of moderate activity towards acetamide was observed in both types of extracts. Attempts to obtain this activity in soluble form for further investigation, by various techniques such as varying the strength of buffer

TABLE III

% Hydrolysis* of amides of lyophilized whole cells of *Candida utilis*

Nitrogen source	Substrate:					
	Acet.	But.	Isobut.	Isoval.	Gly.	Glycine
Acetamide	29.1	14.4	17.3	77.0	25.3†‡	1.0
Yeast extract	35.7	17.3	81.3	61.2	103.4†‡	6.5

NOTE: The reaction mixture contained 60 mg of lyophilized cell powder, 1 ml of 0.1 *M* PO_4 buffer, pH 7.0, 1 ml of 0.1 *M* amide, and 1 ml H_2O. Incubated at 37° for 18 hours in a Dubnoff incubator. Ammonia was determined by the indirect method.

*See definition in Table II.

†Corrected for glycine ammonia.

‡Ammonia determined by direct method only, because of instability of glycinamide to alkaline conditions.

extractions, met with failure. Perhaps this activity is very closely associated with cellular structures, such as the cell membrane. The value of 35.7% hydrolysis for acetamide agrees well with the figure obtained by Gorr and Wagner (2) for the same amide.

Evidence for Plurality of Deamidases

It has been shown that cell-free extracts are capable of deamidating forma-

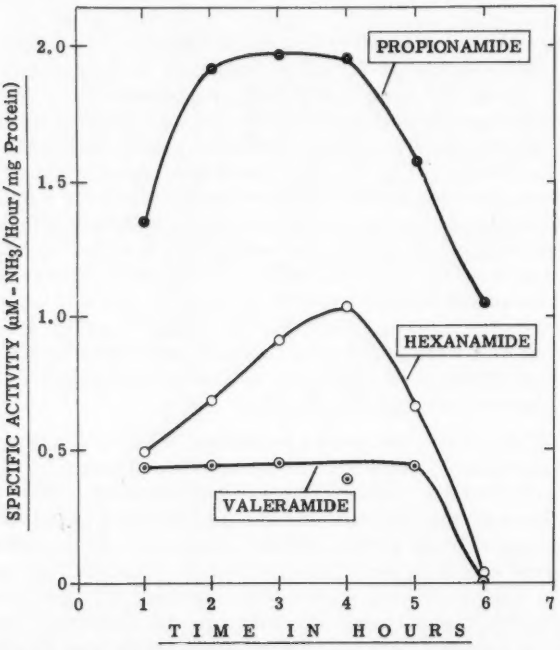

FIG. 2. Variation in specific activities of aliphatic amide deamidases in extracts obtained from whole yeast cells shaken with glass beads for various periods of time. Enzyme assay as described in the text.

TABLE IV

Separation of propionamide deamidase from extracts of *Candida utilis*

Fraction	Units*			Specific activity*		
	Prop.	Val.	Hex.	Prop.	Val.	Hex.
0.1 *M* PO_4 buffer, pH 7.0	363	140	219	12.1	4.65	7.20
1 *M* PO_4 buffer, pH 7.0	475	103	247	8.55	1.85	4.44
1 *M* PO_4 buffer, pH 7.0, extraction	45	0	—	5.00	0	Trace
Total units	883	243	466			
% recovery	93	94	82			

*See definition in Table I.

mide, propionamide, butyramide, valeramide, and hexanamide. Acetamide, isobutyramide, isovaleramide, and glycinamide are deamidated by lyophilized whole-cell extracts.

In another procedure, extracts were prepared according to the method described under the section Purification of Aliphatic Amide Deamidases. Cells were broken for an hour, the supernatant extract retained, and the

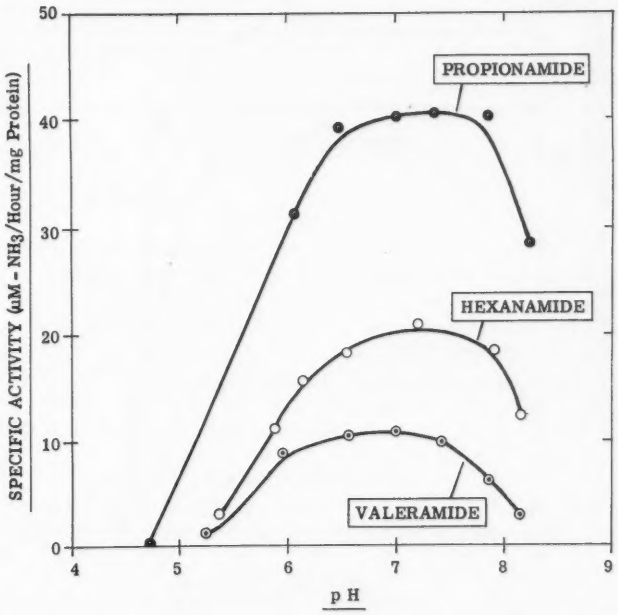

FIG. 3. Effect of pH on propionamide, hexanamide, and valeramide deamidase activities of *C. utilis*. Experimental conditions as described in the text.

unbroken cells and debris resuspended and subjected to further breakage. This cell breakage procedure was repeated as a function of time for several hours. The supernatants from the successive breakages were analyzed for deamidases and the specific activities determined. Typical results of such experiments are shown in Fig. 2. It can be seen that the specific activity ratio propionamide:hexanamide:valeramide is not constant. In fact, extracts were obtained which had activity only with respect to propionamide. It is not, however, excluded that denaturation could be playing a role at this point.

Further evidence concerning the multiplicity or uniqueness of the deamidase activity of extracts of *C. utilis* was obtained by the following procedure: extracts of deamidases were prepared through to step C of the purification procedure (see Table I) previously outlined. The protamine sulphate pellet obtained was successively extracted with phosphate buffer of different strengths. The various extracts so obtained were analyzed for deamidase activities and the results summarized in Table IV. Extracts were obtained which were essentially free of hexanamide and valeramide deamidases but which contained measurable propionamidase activity.

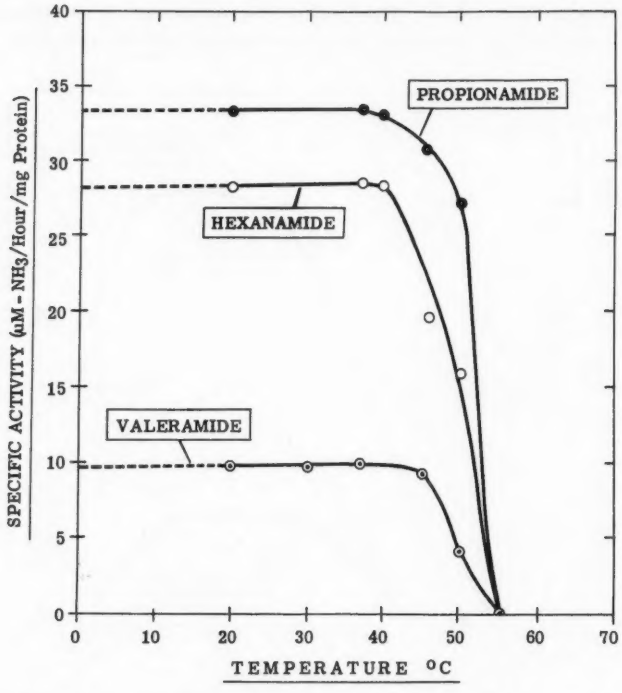

FIG. 4. Thermal denaturation of aliphatic amide deamidases of *C. utilis*. Conditions are described in the text.

Effect of pH on Enzyme Activity

The optimum pH range for the deamidation of propionamide, valeramide, and hexanamide was found to be between 6.7 and 7.8 when run either in Robinson's universal buffer or using the pH stat. (Fig. 3).

Thermal Denaturation of Deamidases

To study the thermal stability of the enzyme, samples were placed in a water bath at the desired temperatures, and held there for 10 minutes. The samples were removed and assayed in the usual manner. Results of such experiments are shown in Fig. 4. All the deamidase activities appear to have the same stability with respect to heat, and all are completely denatured at 55° C.

Effect of Inhibitors

The effect of various types of inhibitors on propionamide deamidase is shown in Table V. Amounts of materials listed in the table were the largest quantities tested.

TABLE V

Effect of inhibitors on propionamide deamidase

Inhibitor	Amount, μM	Specific activity†
None	0	7.8
Chloromercuribenzoate	0.3	7.3
2,4-Dinitrophenol	1.5	8.0
Arsenite*	10	0.9
Arsenate*	10	8.2
Ethylenediamine tetraacetate	6	0
Isoniazid	50	7.5
Iodoacetamide	15	5.2
Cyanide	30	0

*By indirect method only.
†See definition in Table I.

End Products of the Deamidation Reactions

Propionic, valeric, and hexanoic acids have been tentatively identified as end products of propionamide, valeramide, and hexanamide deamidases. Further work is planned in this direction. Glycine has been definitely found as end product of glycinamidase as identified by descending paper chromatography on Whatman No. 1 paper with phenol–water (80:20) and butanol – acetic acid – water (80:20:20) as solvents. Papers were dried and developed with ninhydrin by conventional techniques.

Acknowledgments

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NOTES

NATURAL SYNCHRONIZATION OF A VITAMIN-B_{12}-REQUIRING ARTHROBACTER SP.[1]

I. L. STEVENSON

A number of studies have been reported on a B_{12}-requiring *Arthrobacter* sp. (No. 38) since it was first isolated by Lochhead and Thexton in 1952 (4, 5, 8). Chaplin and Lochhead described the abnormal morphology of this organism resulting from cobalamin deficiency (3). From these early studies and from further investigations conducted in this laboratory it is evident that the deficiency of the vitamin affects the division mechanism of the cell while the growth processes appear unimpeded. As a result, megalomorphism and extremely irregular cells appear in the absence of B_{12}. In continuing studies on the cytological and physiological aspects of growth of this organism attempts were made to synchronize cell division in order to eliminate the usual heterogeneity in an actively dividing culture.

The majority of techniques for synchronizing cell division require the subjection of the bacterial culture to some 'prior treatment' such as a temperature shift or starvation for some essential nutrient (2). These methods are based on physically prolonging one stage of growth, thus allowing the majority of cells in a population to come into one phase. Unrestricted growth after removal of the treatment results in the multiplication of the cells in a synchronous manner. Frequently cultures synchronized by such methods exhibit signs of unbalanced growth attributable to the relatively severe methods used for establishing synchrony. Maruyama and Yanagita (6) devised a filtration technique in which cells were separated according to size, and were able to show that cells of the same size were phased and were capable of dividing synchronously on transfer to fresh medium. This method of achieving synchrony has little effect on the cell, and, using it, McFall and Stent (7) and others were able to demonstrate the continuous synthesis of macromolecules during synchronized growth.

Arthrobacter spp. in general are characterized by an extended lag in cell division when transferred to fresh medium. During this lag the cells increase in size and exhibit the pleomorphism typical of the genus (9). This phase is then followed by cell division. It does not seem unreasonable to assume that a partial phasing of the culture might be taking place during the extended lag and enlargement phase of the growth cycle. Since *Arthrobacter* No. 38 exhibits these typical growth habits, attempts were made to utilize this natural phasing to synchronize the culture.

Arthrobacter No. 38 was cultured routinely on yeast extract broth (5) supplemented with 1 μg vitamin B_{12}/ml. The growth characteristics of this organism on transfer from a maximum stationary phase culture (36-hour)

[1]Contribution No. 522 from Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa.

to fresh medium YB_{12} (shake culture) were as follows: a lag period of 10–12 hours occurred during which time the cells enlarged and exhibited typical pleomorphism followed by exponential division; the maximum stationary phase was reached after approximately 30 hours. The generation time of the organism was equal to 4.5 hours.

In synchronization studies 36-hour stationary phase cultures were centrifuged, washed, and resuspended in fresh medium YB_{12}. Turbidity was adjusted to a Klett reading of 500 at 540 mμ. Two milliliters of this suspension was used to inoculate nepheloculture flasks containing 50 ml of medium YB_{12}. Flasks were incubated in a shaker–water-bath at 28° C for 14 hours to bring the cells just into the exponential phase. These exponential-phase cells were then centrifuged, washed with aliquots of YB_{12}, and brought to a Klett reading of 500 at 540 mμ. Fresh nepheloculture flasks were inoculated with 2 ml of this suspension of exponential-phase cells. Numbers of cells were determined at half-hourly intervals by direct microscopic count (Helber chamber) and by plating on a solidified YB_{12} medium.

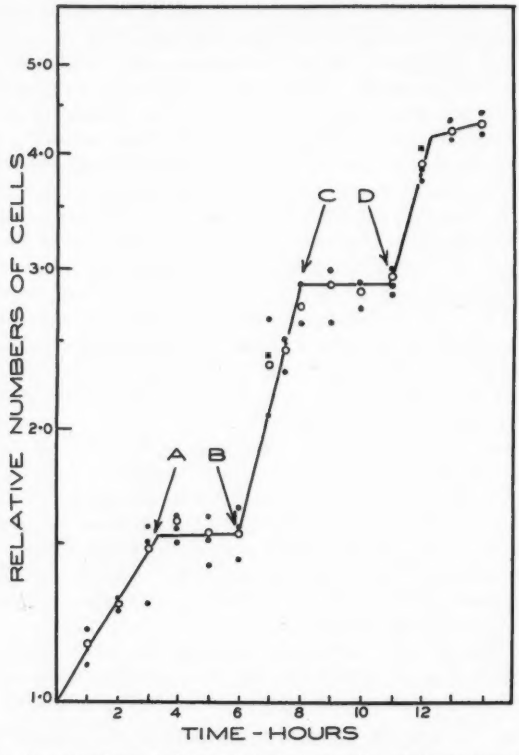

FIG. 1. Synchronized division of *Arthrobacter* No. 38 in medium YB_{12}.

Figure 1 illustrates the results of one such synchronization experiment in which the relative numbers of cells of three replicate flasks are plotted along with their mean value. It is evident from these data that the culture was phased and in the process of completing a division at the time of inoculation. After this first division a plateau is attained where cells have ceased dividing in preparation for the next division. The time required for the cultures to go from the completion of the first division to completion of the second division corresponds to the generation time of the culture (4.5 hours). Synchrony of the culture was lost during the third division. Giemsa-stained preparations of cells sampled after completion of division (positions A and C, Fig. 1) showed that mononucleate organisms were predominant while cells sampled just prior to division (positions B and D) were binucleate and obviously just ready to divide.

Barner and Cohen (1) have previously achieved synchronous growth of a mutant of *Escherichia coli* by withholding thymine for a period of

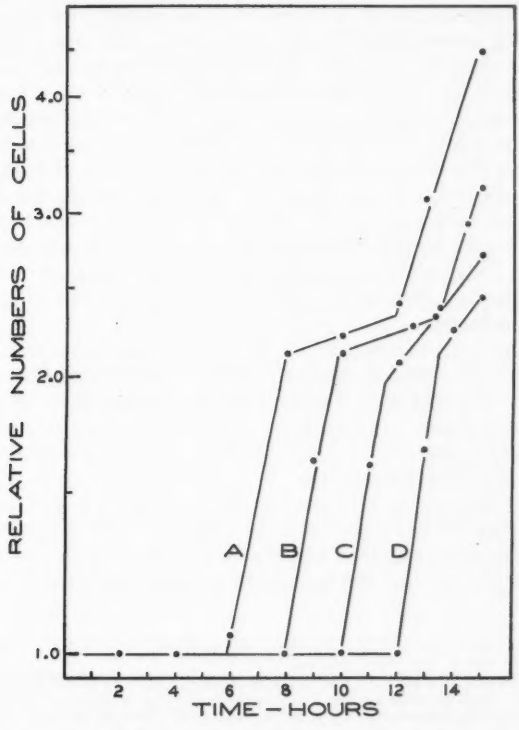

FIG. 2. The effect of withholding vitamin B_{12} from synchronized cultures of *Arthrobacter* No. 38. A, B_{12} added at zero time; B, B_{12} added at 2 hours; C, B_{12} added at 4 hours; D, B_{12} added at 6 hours.

30 minutes. In the absence of thymine the cells failed to divide, but on readdition of the DNA precursor the cells phased and divided synchronously. The similarity between the thymineless mutant of *E. coli* and the B_{12}-requiring *Arthrobacter* prompted a study of the effects of withholding vitamin B_{12} from a culture of *Arthrobacter* No. 38 which had been prepared for synchronous division.

Inoculum was prepared as previously described and 0.5 ml added to nepheloculture flasks of medium Y (5). Vitamin B_{12} (1 μg/ml) was added to duplicate flasks A at time of inoculation, to duplicate flasks B 2 hours after inoculation, to duplicate flasks C 4 hours after inoculation, and to duplicate flasks D 6 hours after inoculation. All flasks were incubated in a shaker-water-bath at 28° C and sampled at half-hourly intervals for direct microscopic counts and plating.

Figure 2 illustrates the results of these experiments. It can be seen from these data that the inoculum had just completed a division at the time of inoculation as shown by the 6-hour lag prior to cell division in the series to which B_{12} had been added at 0 hours (A). No reason can be given for the length of this lag other than the possibility that cells are affected more on transfer to fresh medium just after division than during division, as noted in Fig. 1. In series A the cells have completed division by 8 hours, which is followed by a second division commencing at 12 hours. Where vitamin B_{12} had been withheld for 2 hours after the start of the experiment (series B), the course of cell division paralleled that of series A except that division was delayed by a period of 2 hours. Similarly, withholding B_{12} from the cultures for 4 and 6 hours (series C and D) further delayed the onset of division by these time intervals. It will be noted in Fig. 2 that synchrony was lost in cultures C and D. Deprivation of B_{12} for the extended periods of 4 and 6 hours apparently has affected the cells in such a way that the cultures are no longer synchronized.

Growth studies on *Arthrobacter* No. 38 have shown that a degree of natural synchrony can be achieved by careful cultural preparation. Since cell enlargement of the coccoid forms of *Arthrobacter* is a prerequisite to division, it is felt that a natural phasing is occurring. At the same time the long generation time of *Arthrobacter* No. 38 (4.5 hours) enables one to manipulate and observe the culture to determine synchrony.

Withholding vitamin B_{12} for varying intervals from synchronized cultures of *Arthrobacter* No. 38 indicated that B_{12} affects the division processes of the cells in a manner similar to that of thymine for the *E. coli* mutant of Barner and Cohen (1). Continuing studies on the role of vitamin B_{12} in cell division are in progress.

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A SIMPLIFIED METHOD FOR DETERMINING THE SENSITIVITY OF ANAEROBIC BACTERIA TO ANTIBIOTICS

B. MARTINEAU AND V. FREDETTE

Introduction

A few methods have been published concerning the determination of the sensitivity of anaerobic bacteria to antibiotics, particularly with the help of paper disks already in use for aerobic bacteria.

In 1954, Beerens and Guillaume (1) published a technique which calls for: 1, the preparation of a short column of Deep Agar in 180×12- to 180×13-mm tubes; 2, the deposition of a paper disk; 3, affixing this paper disk with sterile agar; 4, adding about ten milliliters of inoculated agar. Such a method requires the opening of the tubes at least four times for a single determination.

A method also used in France requires attaching with sterile agar four or five paper disks in the sterile cover of an ordinary Petri dish, then pouring 15–20 ml of inoculated agar, and finally pressing the outside bottom of a Petri dish into this agar and sealing off the exposed agar with molten paraffin (6).

Having in mind the routine work of a hospital laboratory where simple, uncomplicated techniques requiring standard materials are necessary, we endeavored to combine the advantages of the paper disk technique with the use of Deep Agar favored by French and German bacteriologists for the isolation of the anaerobes. The relative merits of this latter technique and that of the anaerobic jar method may account for the fact that Prévot describes more than 480 species (5) while Bergey mentions only 160 (2).

Technique

In its present form, the technique is quite simple. It consists only in heavily seeding regular tubes of Prévot's Deep Agar in Veillon tubes (10 mm × 150 mm), then suspending individual antibiotic-containing paper disks in the middle of each contaminated agar column.

(*A*) *Regeneration of the Agar*

The Deep Agar (6) must first be melted and deaerated. According to Prévot's method (6), this can be accomplished by placing the tubes in boiling water for 20–30 minutes. But Fredette and Takahashi (3) have found that a 5-minute steam bath in the unpressurized autoclave will give an equally satisfactory reduction of the rH potential, as far as could be ascertained by the use of Methylene Blue, Janus Green, Cresyl Violet, and other rH indicators in the range 14 to 7.4 (Eh, -0.005 to -0.250).

Once deaerated, the melted agar can be kept in the water bath at 45° C until ready to be seeded. After 24 hours, regeneration again becomes necessary, but a given lot of agar should never be submitted to this treatment more than twice.

(*B*) *Seeding the Agar*

The goal here is to obtain a uniform distribution of the bacteria in the inoculum. This is attained easily by dipping the tapered portion of a Pasteur pipette into a broth culture of the test organisms, then pushing it to the bottom of the tube of molten agar, whereupon it is rotated between thumb and forefinger at least 10 times. This procedure must be repeated for each individual tube of agar, using either a closed or an open Pasteur pipette (for lightly growing cultures).

The seeded tubes of agar are replaced in the water bath until the operator is ready to proceed with the next step.

(*C*) *Suspending the Antibiotic Disks*

The unique feature of the method resides in placing the seeded tubes in cold water (ice in summer) to about half their height, then dropping individual paper disks in each tube. Slight tapping will cause the disk to sink into the agar until it stops by itself when it reaches the solidified portion of the agar halfway down the length of the agar column.

The remainder of the agar column is made to solidify rapidly to prevent undue diffusion in the still-liquid portion. The tubes may then be transferred to the incubator.

Results

Results may be read in less than 24 hours in the majority of cases, for some bacteria as early as 8–12 hours, i.e. in a single day's work. They may be expressed as width (in millimeters) of the zone of inhibition (including its extent on both sides of the paper disk), but hospital bacteriologists may prefer to use the + to ++++ system.

Figure 1 shows what can be expected in the case of an organism producing no gas, such as *Clostridium histolyticum*, which is the easiest case to read.

In the case of *Clostridium perfringens* or of any other gas-producing microorganism, readings should be taken at 8 hours or at any time when growth is already visible but no gas has yet evolved.

Discussion

The main feature of this new technique lies in the fact that, since microorganisms are to be found on both sides of the paper disk, there is no uncer-

PLATE I

FIG. 1.

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tainty as to whether the agar was properly seeded. Moreover, the strict anaerobic character of the culture under test is shown simultaneously.

Finally, there is no need for special materials or extra manipulations; the tubes and techniques are exactly the same as those already in use for isolation of anaerobic bacteria according to Prévot's method. At any rate, it seems to us that the present modification is simpler than that published by Beerens and Guillaume (1), in which the poor keeping-qualities of the short column of agar make it imperative to have fresh media daily. As mentioned previously, this older technique requires the tubes to be opened at least four times whereas here they are opened only twice.

We have made a few tests with Difco's Dextrose Broth Infusion Agar with good results, but we cannot at this time state whether this medium is equal to Prévot's Deep Agar for growing the more fastidious species of anaerobes (6).

Résumé

Une méthode simplifiée, qui combine les avantages de la gélose profonde de Prévot et ceux des disques séchés d'antibiotiques, a été mise au point pour la détermination de la sensibilité des bactéries anaérobies aux antibiotiques.

Elle comporte des avantages quant à la préparation et à la conservation des milieux; en outre, elle réduit de moitié les manipulations requises par les modifications antérieures de cette technique.

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EFFECT OF SEITZ FILTRATION ON EXTRACELLULAR STAPHYLOCOAGULASE[1]

HAMDI A. TAMIMI

Extracellular staphylocoagulase has been shown to pass through Seitz filter pads (2). However, the literature contains little information on the amounts of coagulase lost through filtration of coagulase-positive cultures and solutions of partially purified coagulase preparations.

Partially Purified Coagulase Preparation

One milliliter of a 24-hour broth culture of a potent coagulase-positive staphylococcal strain previously isolated from a fatal case of staphylococcal septicemia was seeded in approximately 800 ml of sterile Difco brain heart infusion (BHI) broth and incubated for 48 hours at 37° C. Cells were removed by centrifugation at 300 r.p.m. for 30 minutes. Three volumes of 95% ethyl alcohol was added to the supernatant fluid according to the method of Gerheim *et al.* (1). The resulting precipitate was removed by decantation and dissolved in approximately 100 ml of BHI broth. It was reprecipitated by 3 volumes of alcohol, dried, and stored in a desiccator. The dry coagulase was dissolved in 50 ml of sterile BHI broth adjusted to the same pH as that of a 24-hour culture of the donor strain. Five arbitrary dilutions were made in the same broth. A portion of each dilution was passed through a Seitz filter pad and all 10 portions were assayed for coagulase contents by the procedure subsequently described.

Cultures

Eleven coagulase-positive staphylococcal strains isolated from various pathological conditions were subcultured in 25 ml of BHI broth in 100-ml Erlenmeyer flasks and incubated for 22–24 hours at 37° C. Each culture was passed through a Seitz filter pad and the sterile filtrates were titrated for coagulase content. A portion of each filtrate was refiltered and again titrated for coagulase content. Coagulase titer of the first filtrate was compared with that of the scond.

Coagulase Test and Titration

The test was performed by pipetting 0.5 ml of the material tested into a Wassermann tube. Each tube received 0.5 ml of normal rabbit plasma diluted 1:4 with saline, and incubated at 37° C for 24 hours.

Titration for coagulase content of culture filtrates was performed by the twofold serial method. BHI broth was the diluent. The same aliquot of diluted plasma was added to all tubes and incubated as before.

The 10 portions of the partially purified coagulase preparation were titrated as follows: 1-ml aliquots of each dilution were pipetted into an appropriate number[2] of Erlenmeyer flasks containing sterile BHI broth. The volume of

[1]Supported by a grant from the United States Public Health Service.

[2]Nine flasks for the highest dilution, 18 flasks for the lowest dilution, and an intermediate number of flasks for the other dilutions.

broth in each flask ranged between 49 and 499 ml. The contents of the flasks were mixed by agitation. From each flask, 0.5 ml was removed to a Wassermann tube. Each tube then received 0.5 ml of diluted plasma and was incubated as before.

Each experiment was repeated at least three times. Controls were included with every experiment. Only tubes containing organized clots equal to 2+ or more (3) were considered positive for coagulase.

TABLE I

Effect of Seitz filtration on coagulase titer of partially purified coagulase solutions

Coagulase titer*		No. of determinations	% activity lost
Control portion	Filtered portion		
430±8.56†	340±9.66	6	23.3
340±7.74	280±8.56	6	17.6
250±10.0	210±8.36	5	16.0
170±6.37	140±5.78	6	17.7
85±4.41	80±2.88	4	5.9

*Highest dilution yielding ++ clot in plasma.
†Standard error of mean.

Percentage coagulase activity lost by filtration of partially purified coagulase solutions is shown in Table I. Approximately 25% was removed from the solution containing a titer of 1:430 as compared with only 5.9% from that containing 1:85.

Seitz filtrates of the 11 coagulase-positive staphylococcal cultures gave a positive test for coagulase, as titers ranging from 1:4 to 1:32 were obtained (Table II). Little or no coagulase activity was removed from these filtrates when they were filtered again.

TABLE II

Coagulase titration of 11 coagulase-positive cultures using Seitz filtrates of the cultures

Strain No.	Coagulase titer				No. of determinations
	First Seitz filtrate*	Degree of clot	Second Seitz filtrate*	Degree of clot	
2 and 9	1:16	++	1:8	+++	4 and 3
3, 5, and 6	1:4	++	1:4	++	3, 3, and 5
4	1:4	++	1:4	+++	6
7	1:32	+++	1:16	+++	3
8 and 12	1:16	++	1:16	++	3 and 3
10 and 11	1:8	++	1:8	++	3 and 3

*The first Seitz filtrate was obtained by passing broth culture through a Seitz filter pad. The second Seitz filtrate was obtained by passing a portion of the first filtrate through another Seitz filter pad.

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HAPLOMYCOSIS (ADIASPIROMYCOSIS) IN SOREX[1]

A. BAKERSPIGEL

Haplosporangium has been observed in, or isolated in cultures from, the infected lungs of many small mammals. Reports of these isolations have come from several countries including the United States and Canada (1, 2, 3, 4).

The present report records the finding of the spherules (adiaspores) of *Haplosporangium* in the lungs of an additional mammalian host. This was a shrew trapped in 1957 near Lloydminster, Alberta, Canada. Figures 1 and 2 show the characteristic spherules in sections of the infected lungs of this shrew. The species of *Haplosporangium* in this tiny mammal appears to be similar to the Alberta strains described previously by Dowding (3) and Bakerspigel (1, 2) as well as the fungus *Emmonsia crescens* Emmons & Jellison (4).

Tevis (5) previously reported finding *H. parvum* in the lungs of *Sorex araneus* and *S. minutus* in Dorset, England, but this is the first record of *Haplosporangium* in *Sorex* in North America.

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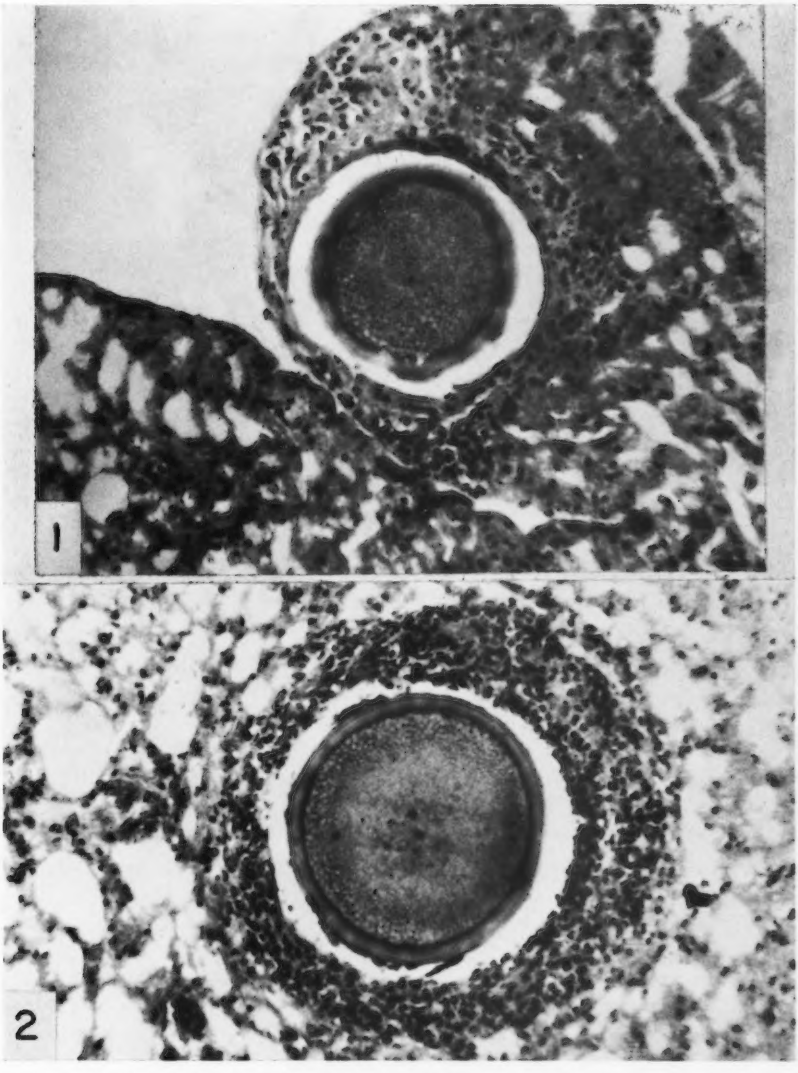
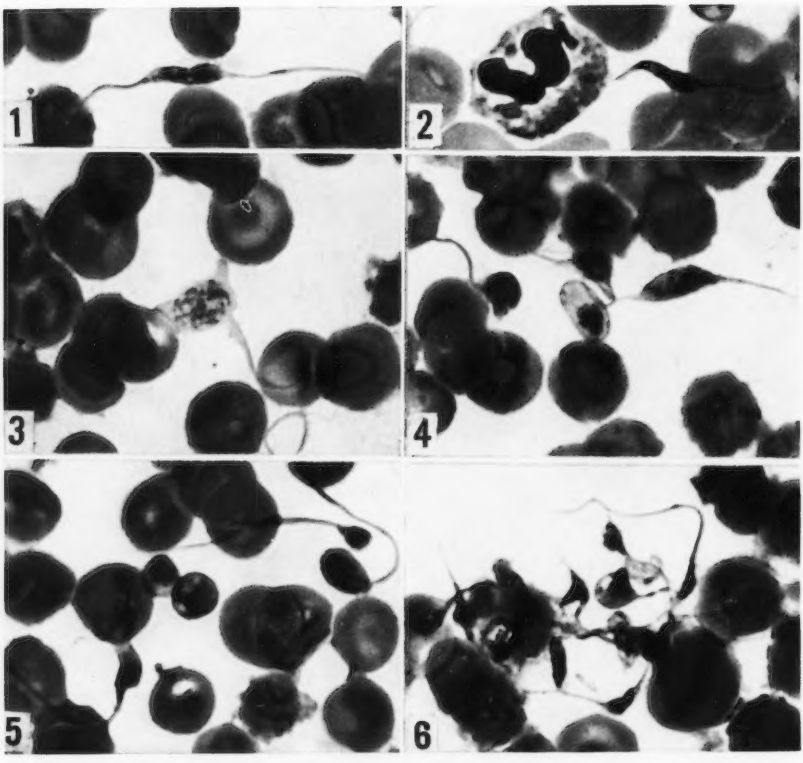


FIGS. 1 and 2. Spherules (adiaspores) of *Haplosporangium* in sections of infected lung from a shrew (*Sorex* sp.). Hematoxylin and eosin, ×225.



FIGS. 1–6. Pseudoflagellates in canine blood, ×1650 (approx.). (Enlarged from negatives taken at 850 diameters.) Figs. 1 and 2. Pseudoflagellate forms. Fig. 3. Large platelet with pseudopodia and flagellum. Fig. 4. Normal platelet and flagellate forms. Figs. 5 and 6. Irregular, flagellate, and ciliated forms.

PSEUDOFLAGELLATES IN FILMS OF CANINE BLOOD

A. SAVAGE AND J. M. ISA

In March, 1961 we received a stained film of dog blood. Under the microscope it showed a number of objects many of which suggested minute flagellates. As shown in Plate I, their over-all appearance was not uniform. Each consisted of a "body" approximately the size of a blood platelet. This contained no recognizable nucleus but only a number of blue granules. There were one or two appendages that varied up to about 10 μ in length. Most of these were tapered, others clearly filiform. In some cases they arose from opposite sides of the body (Figs. 1, 2, and 6), the resulting forms suggesting tiny trypanosomes. A few cells were found that showed extrusions resembling pseudopodia in addition to a long flagellum (Fig. 3).

These forms occurred in groups rather than at random. That fact, their size, their staining reaction, and their common association with blood platelets indicate that they are artifacts derived from platelets.

The manner of their derivation is speculative. More films were obtained from the same dog with the unexpected result that in some of them these pseudoflagellates abounded while in others they could not be found! We have been unable to reproduce them by using the blood of other dogs and slides cleaned with various detergents. Unfortunately for the pursuit of this curious phenomenon, the dog concerned has left the country at least temporarily. Thus, while our pictures may add something to Tocantins' comprehensive paper (1), the case as such remains an isolated curiosity.

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ARREST OF BACTERIAL SPORES IN STAGES OF POSTGERMINATIVE DEVELOPMENT[1]

MANUEL GOLDMAN AND HAROLD J. BLUMENTHAL

During postgerminative development of *Bacillus megaterium* in a synthetic medium, changes in the respiratory rate accompany the morphological changes leading to the formation of the first pair of vegetative cells. Some of these developmental stages have specific characteristics, such as requirements for sulphur or phosphorus compounds, or a susceptibility to the blocking of further development by the presence of certain metals (6, 7). Other germinated bacillary spores, including *B. cereus*, require a number of amino acids for

[1]Supported in part by a grant from the United States Public Health Service.

postgerminative development (2, 8) but can develop synchronously into vegetative cells in a medium containing glucose and protein hydrolyzate (3, 9). While studying glucose metabolism in intact *B. cereus* spores (1, 4), we found it necessary to prepare uniform spore suspensions, in various stages of postgerminative development, that were incapable of developing further even while oxidizing glucose in a buffered medium for as long as 4 hours. The present report is concerned with a principle for arresting suspensions in any stage of synchronous, postgerminative development, without the necessity of adding inhibitors or determining the organism's exact nutritional requirements.

Generally, 0.5 g of clean, lyophilized *B. cereus* strain terminalis spores, grown in a modified G. medium (5), were suspended in 10 ml of 0.05 *M* sodium phosphate buffer, pH 7.0, and heated at 65° C for 1 hour. Germination of the heat-activated spores was completed within 3 minutes after the addition of 240 μmoles of adenosine and 100 μmoles of L-alanine, and aeration at 30° C on a rotary shaker (160 r.p.m.). The germinated spore suspension, which would not develop further if allowed to remain in the germination medium, was then added to a 2-liter Erlenmeyer flask containing 600 ml of temperature-equilibrated Trypticase–soy broth (Baltimore Biological Laboratories) and placed on a rotary shaker at 30° C. The time-course of postgerminative development was followed by removing samples and observing them with phase contrast optics. At the particular stage of development selected, the cells were rapidly chilled and centrifuged for 10 minutes at 10,000 *g* in the cold. After the supernatant fluid was decanted, the packed cells were dispersed in 100 ml of cold 0.05 *M* sodium phosphate, pH 7.0, and recentrifuged. This procedure was repeated once again and the washed cell pack was resuspended in fresh buffer and used as desired. If the germinated spores were left to incubate in the Trypticase–soy broth, it took 120 minutes for them to complete the first cell division synchronously.

When spore suspensions, arrested in any stage of postgerminative development, were incubated with shaking for 4 hours at 30° C in the presence of 0.003 *M* glucose, there were no detectable morphological changes in the cells. Spores in the late stages of elongation shed their coats during the prolonged period of incubation but the spores themselves were otherwise not visibly changed (Figs. 1 and 2). Also, these cell suspensions, actively utilizing glucose, remained metabolically distinct at any stage of development with respect to the pathways of glucose catabolism (1) and were completely viable even after 4 hours of incubation. They would resume their normal development within a few minutes after replacement in a Trypticase–soy medium. Viability was measured by placing two loopfuls of Trypticase–soy agar on a glass slide already containing a loopful of the incubated flask contents, mixing, covering with a cover slip, and observing with phase contrast optics. At least 500 spores in five fields were observed, and all of them could be seen to have completed at least two cell divisions.

Although these studies were performed with a single strain of *B. cereus*, preliminary experiments with spores of *B. subtilis* strain globigii gave similar results and it seems probable that spores of other aerobic bacilli may also be

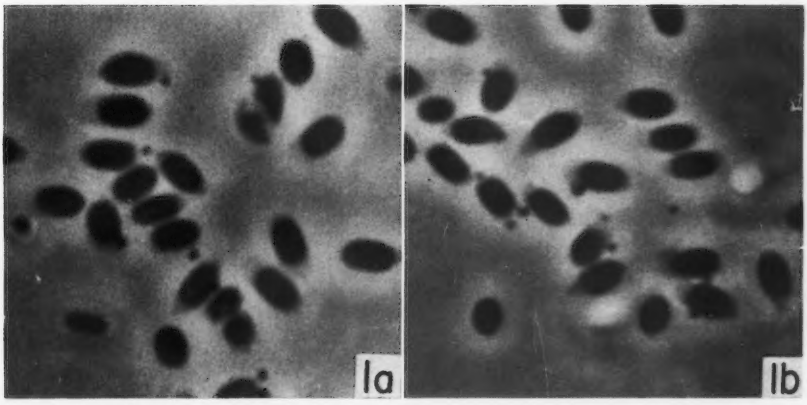

FIG. 1. Effect of extended aeration in a glucose–phosphate medium on the morphology of spores arrested, after germination, in the swollen stage: (*a*) prior to aeration; (*b*) after aeration for 4 hours at 30° C on a rotary shaker (160 r.p.m.).

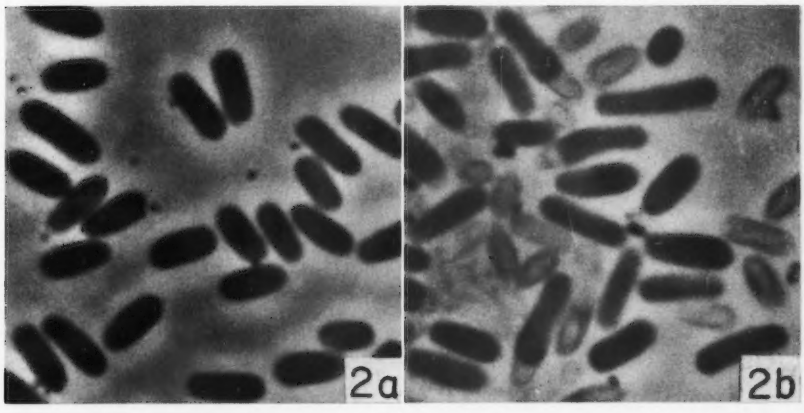

FIG 2. Effect of extended aeration in a glucose–phosphate medium on the morphology of spores arrested, after germination, in the late elongation stage: (*a*) prior to aeration; (*b*) after aeration for 4 hours at 30° C on a rotary shaker (160 r.p.m.).

studied in the same manner. The availability of spore suspensions stabilized in any of the stages of postgerminative development should aid studies relating morphological and metabolic differentiation during the development of a spore into vegetative cells.

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